ANNUAL REVIEW OF BIOCHEMISTRY

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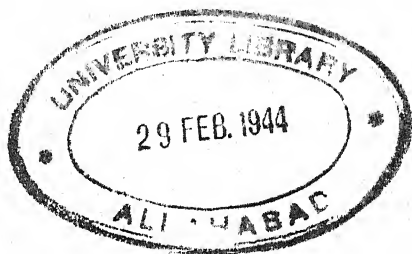
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PREFACE

The preface to the preceding volume was written on April 9, 1940. By a singular coincidence the present paragraphs are being written on a comparable day in 1941 as the expanding forces of war reach into another European nation. The occasion is not one that would call for comment in these pages were it not that the war, far-reaching in its ramifications, has profoundly influenced not only scientific research but every field of learning. Many of the universities and scientific institutes of continental Europe are closed. In many others scholarly activities are virtually in abeyance. Libraries have been completely destroyed. Several scientific journals have suspended publication. The current publications of one continent or, in some cases, of a neighboring country arrive with disturbing irregularity, if at all. Scientific research confronted by the exigencies of the moment, is changing in its complexion and emphasis. Many have abandoned, by choice or duress, any attempt to engage in experimental work in the pure sciences for the duration of the emergency.

In so far as the present volume of the *Review* is concerned, it may appear on cursory inspection, that the war has had but little effect. It is true, for example, that the world-wide literature of biochemistry has contracted but little: the number of papers calling for review still exceeds by far the amount of space that may wisely be devoted to the various subjects. It is equally true that the international character of the *Review* is not entirely lost: our colleagues abroad, wherever collaboration in authorship continues to be possible, have been most generous in giving their assistance to the present task by writing reviews. In Volume XI the international character of the *Review* will be more manifest than in Volume X.

The most serious impact of the war upon the *Review* might escape the observation of many. Delays and irregularities in the receipt of journals have been numerous. Almost every article in the present volume has been submitted by its author with apologies for the restrictions in content imposed by the unavailability of recent numbers of important journals. In some cases a solution to the problem has been approached, though not with entire satisfaction, by the increased use of published abstracts for review purposes and by the solicitation of reprints of recent papers. We need hardly mention that reprints will be most gratefully received, especially in those cases where there

is reason to suspect that the journal of publication may not be accessible to the reviewers.

To all who have assisted in the preparation of the present volume, especially to the authors, we wish to express our most sincere thanks. Suggestions in respect to authorship and subject matter continue to be received from many and are ever welcome. Professor H. S. Loring assisted us greatly in preparing the manuscripts for press. To him, to our editorial assistants, and to the Stanford University Press we wish to express our gratitude for the most cordial co-operation.

A cumulative author and subject index to Volumes I to X is now in preparation and will be published in the autumn of 1941.

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J. M. L.
H. J. A.
D. R. H.
C. L. A. S.
J. H. C. S.
H. A. S.

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IN MEMORIAM

Carl Lucas Alsberg died in Berkeley on October 31, 1940. Death was due to pneumonia, contracted in New York, where he was engaged in important conferences dealing with utilization of natural resources in national defense.

Alsberg was born in New York in 1877. His father was a chemist and he grew up in the atmosphere of science. Undergraduate work was completed in Columbia University in 1896, followed by the Master's degree in 1900, at which time he also took the degree in medicine. Then followed three years of study abroad, mostly in Strasbourg and Berlin; it was in Strasbourg particularly that his exceptional talent was first recognized. Returning to this country, he became instructor in biological chemistry in Harvard, where he remained until 1908. He then went to Washington as chemical biologist to the Bureau of Plant Industry. As early as 1912 he was promoted to the position of Chief of the Bureau of Chemistry, which he retained until 1921. He modernized this department. In the period just preceding and during the war, he was a tower of strength in the Department of Agriculture, the man upon whom Secretary Houston relied more than on anyone else, as the writer knows from repeated personal statements of the Secretary. In 1921 he was one of the three directors selected to open the recently endowed and established Food Research Institute in Stanford University. In 1937, he was induced to accept the Directorship of the Giannini Foundation of Agricultural Economics in the University of California, which position he occupied at his death. Alsberg was a man of extraordinary qualities and capacities. He had the invaluable combination of memory and curiosity, and covered both fields of biological and social science. While in Washington, he made an intensive investigation of Pacific Fisheries. In Stanford University his interest broadened to include all phases of intercourse between the populations of Asia and the Western Hemisphere. Under these circumstances, it was inevitable that he became a prominent factor in the Institute of Pacific Relations, and extended his personal investigations to Asiatic conditions. It was directly corollary to these international relations that in the '30's his interest became active in social sciences.

At the time of his death, few men on the Pacific Coast possessed his breadth of interest and extensive experience. Alsberg could always

be counted on to carry more than his share of a load. He was from the beginning associated with the *Annual Review of Biochemistry;* in this activity, as in other directions, he sought to make the records of science so comprehensive as to be available to all workers. Himself possessing an encyclopedic memory, he sought on the printed page to have similar facilities made available to all.

A most notable contribution made by Alsberg to California was in the field of postgraduate instruction. In an almost literal sense, Alsberg had no experience in undergraduate instruction; yet in Stanford University and the University of California he displayed very unusual judgment in the administration and development of postgraduate instruction. One has but to question President Wilbur and President Sproul to have this emphasized. Probably no man since the World War did as much to elevate postgraduate standards in California as did Alsberg.

In personal life, Alsberg was simple, direct, sincere, and moderate. An unfailing sense of humor endeared him to all his colleagues. His outstanding characteristics were toleration, co-operation, and generosity. He had a deep sense of equity which in all relations—personal and academic, in administration and research—brought co-operative efforts to a high plane. His death in his early 60's means to biological science in the United States the unfulfillment of a natural promise of a decade of activity. To California it means the untimely loss of a distinguished leader.

ALONZO E. TAYLOR

ERRATA

Volume   IV, page 476, line 8 from bottom: *for* aërobic, *read* anaërobic.

Volume VIII, page 198, line 14 from bottom: *for* lysozome, *read* lysozyme.

Volume   IX, page   84, line 20: *for* 4-β-*d*-galactosido-*d*-glucose, *read* 4-β-*d*-galactosido-*d*-fructose.

Page 208, line 15: *for* $(CH_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot COO)_2 Hg \cdot (HgCl_2)_4$, *read* $[CH_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COO]_2 Hg \cdot (HgCl_2)_4$.

Page 287, line 1: *for* [1(+)-abrine], *read* [*l*(+)-abrine].

Page 406, line 13: *for* dipheriae, *read* diphtheriae.

Page 430, line 4 from bottom: *for* these, *read* the latter.

Page 594, line 9 from bottom: *for* originally, *read* later.

Page 596, line 22: *for* and, *read* and later.

Page 673, line 10: *for* Blanck, *read* Blauch.

Page 687, reference 94: *for* Blanck, *read* Blauch.

Page 693, column 1, line 20: *delete* Blanck, M. B., 673.

Page 693, column 1, line 25: *after* Blauch, M. B., 309, 596, *add* 673.

CONTENTS

BIOLOGICAL OXIDATIONS AND REDUCTIONS[1]

By E. S. Guzman Barron

The Lasker Foundation for Medical Research and the Department of Medicine, University of Chicago, Chicago, Illinois

In the last few years the subject of biological oxidations and reductions has made important advances. Initial work with the inhibitor and artificial catalysis technique had already pointed out that oxidation-reduction enzymes were in reality complex systems made up of a number of components. The isolation of some of these systems has confirmed the assumption. It is now generally agreed that biological oxidations are performed through the agency of a specific activating protein and a number of reversible oxidation-reduction systems which transfer electrons, in a series of graded steps, from the oxidizable substrate to molecular oxygen. However, there are still left signs of the old controversy between the followers of the Thunberg-Wieland school and of the Warburg school, remnants which have entrenched themselves in the terminology. As happens with every branch of science, the methods used by a few leaders (in this case, Warburg and his co-workers in their brilliant work on the isolation of the components of enzyme systems) were soon over-diligently applied elsewhere and a great number of papers were published during 1938 and 1939. The present year has been relatively quiet. It is, therefore, a propitious time to take stock of the recent rapid advances and discuss their possible application to the living cell.

ELECTROMOTIVELY ACTIVE SYSTEMS

Metalloporphyrins.—Steady though slow progress is being made in our knowledge of metalloporphyrins, which are the last link for the transfer of electrons to molecular oxygen.

Clark and his co-workers (38, 39) have made a fundamental contribution to our understanding of the equilibrium reactions of these substances and their nitrogenous coordination compounds. The nomenclature proposed by Clark is followed here because of its simplicity. A compound formed from a porphyrin and a metal ion is called a metalloporphyrin. The metal and its state are designated by

expressions such as ferro, cobalti (ferroprotoporphyrin, cobaltimeso-porphyrin). A compound formed from a metalloporphyrin and another substance is designated by prefixing the name of the coordinating substance of the metalloporphyrin, as in cyanide ferriprotoporphyrin (this compound having been called also cyanide parahematin and cyanide ferrihemochromogen).

Since porphyrin, metalloporphyrin coordinating base, and base metalloporphyrin may have distinctive, or, in some cases, common groups with different acidic dissociation constants, there are numerous theoretical possibilities for the integration of the free energies of acid ionization and of hydrion dilution with the free energy of the ideal oxidation-reduction process. Actually, all may be integrated in what Clark (36) calls a continuum. A number of factors which make up the continuum have been discussed by Clark and his co-workers. By postulating the several species found in the following equations of equilibrium[2]

$$K_r = \frac{[Rm]\,[B]^2}{[B_2\,Rm]}; \qquad K_{o_1} = \frac{[BO]\,[B]}{[B_2O]}; \qquad K_{o_2} = \frac{[O]\,[B]}{[BO]};$$

$$K_{h_1} = \frac{[O\,\overline{OH}]}{[O]}; \qquad K_{h_2} = \frac{[O\,\overline{OH}]}{[O]};$$

$$K_{h_2} = \frac{[B_2\,O\,\overline{OH}]\,[H^+]}{[B_2O]}; \qquad K_{h_3} = \frac{[BO\,\overline{OH}]\,[H^+]}{[BO]}$$

they derive the equation for the oxidation-reduction potential:

$$E_h = E_o + \frac{RT}{nF} \ln \frac{[S_o]}{[S_r]} + \frac{RT}{nF} \ln \frac{K_{o_1}K_{o_2}}{K_r} + \frac{RT}{nF} \ln$$

$$\frac{[H^+]\,(K_r + [B]^2)}{K_{o_1}K_{o_2}\,([H^+] + K_{h_1}) + K_{o_1}\,[B]\,([H^+] + K_{h_3}) + [B]^2\,([H^+] + K_{h_2})}.$$

[2] $K_r =$ dissociation constant of reduced base metalloporphyrin; $K_o =$ dissociation constant of oxidized base metalloporphyrin; $[B] =$ molar concentration of free, uncombined, coordinating base; $[Rm] =$ molar concentration of reduced metalloporphyrin; $[O] =$ molar concentration of oxidized metalloporphyrin; $[O\,\overline{OH}] =$ molar concentration of oxidized metalloporphyrin associated with 1 mole of hydroxyl ions per mole; $E_h =$ electrode potential referred to the hydrogen standard; $E_o =$ a constant as defined by Clark (37); $R =$ gas constant; $T =$ absolute temperature; $n =$ number of electrons; $F =$ the faraday; $S_o =$ sum of apparent molar concentrations of oxidized metalloporphyrin and oxidized base metalloporphyrins; $S_r =$ sum of apparent molar concentration of reduced metalloporphyrin and reduced base metalloporphyrins.

When $[H^+]$ is much greater than K_{h_1}, K_{h_2}, and K_{h_3}, and the value of $[B]$ is constant, the slope will be $-\dfrac{dE_h}{dpH} = 0$. The cyanide system studied by Barron (15), Davies (48), and Vestling (145) has this slope. K_{h_2} must be much less than $[H^+]$. In other words, the introduction of the negatively changed cyanide ions repels OH^- even at high concentrations of the latter.

When $[H^+]$ is much less than K_{h_1}, and K_{h_2}, and K_{h_3}, and the value of $[B]$ is constant, the slope will be $-\dfrac{dE_h}{dpH} = 0.06$. The systems pyridine, α-picoline, nicotine protoporphyrin (15), coproporphyrin, etioporphyrin (145), mesoporphyrin, and hematoporphyrin (48), showed this slope.

When $[B]$ is 0, $-\dfrac{dE_h}{dpH} = -0.06$; such a slope was found by Barron (15) in the potentiometric titration of iron-protoporphyrin and iron-spirographisporphyrin.

One conclusion that arises from a consideration of these systems and which is implicit in the equations is that the change of potential accompanying the addition of coordinating base to a fixed mixture of the reduced and oxidized species of the metalloporphyrin is a measure of the ratio of the two dissociation constants. This is of great significance for an understanding of the varied catalytic effects of the different oxidation-reduction systems which form part of the enzyme systems. Indeed, identical conditions are found when phosphopyridine nucleotides (di- and tri-) combine with different activating proteins or when flavins (mono- and dinucleotides) combine with proteins. The potentials of these complexes must change, at constant pH, according to the value of the dissociation constants.

On studying the potentials of iron-proto- and spirographisporphyrin and their nitrogenous compounds, Barron (16) found that the potentials of the latter were always more positive than those of protoporphyrin, a phenomenon of biological significance because, according to Warburg's (147) photochemical measurements, the spectrum of the carbon monoxide compound of cytochrome oxidase (*Sauerstoffübertragendes Ferment der Atmung*) resembles the spectrum of the carbon monoxide compound of spirographis hemoglobin. Table I shows data compiled from the publications of Taylor (139), Davies (48), Vestling (145), and Barron (16). Like the potentials of quinones, the potentials of iron-porphyrins change on substitution of certain groups

in the pyrrol nucleus. The data have been assembled in this table to draw attention to this phenomenon, which has not yet received adequate attention.

TABLE I*

OXIDATION-REDUCTION POTENTIALS OF SOME NITROGENOUS
METALLOPORPHYRIN COMPOUNDS†

Nitrogenous Compound	Metal	Porphyrin	E'_0
			volts
Cyanide	Fe	spirographis porphyrin	−0.113
Cyanide	Fe	protoporphyrin	−0.183
Cyanide	Fe	hematoporphyrin	−0.200
Cyanide	Fe	mesoporphyrin	−0.229
Cyanide	Fe	coproporphyrin	−0.247
Picoline	Fe	spirographis porphyrin	−0.010
Picoline	Fe	protoporphyrin	−0.033
Picoline	Fe	hematoporphyrin	−0.099
Picoline	Co	mesoporphyrin	−0.185
Picoline	Mn	mesoporphyrin	−0.296
Pyridine	Fe	protoporphyrin	+0.015
Pyridine	Fe	hematoporphyrin	+0.004
Pyridine	Fe	etioporphyrin	−0.029
Pyridine	Fe	coproporphyrin	−0.036
Pyridine	Fe	mesoporphyrin	−0.063
Pyridine	Co	mesoporphyrin	−0.265
Pyridine	Mn	mesoporphyrin	−0.387

* Taken from data published by Barron (15, 16), Davies (48), Taylor (139), and Vestling (145).
† pH 9.63, Temp. 30°.

The quantitative treatment of the equilibrium reactions between a nitrogenous base and metalloporphyrin is complicated by the effect of possible polymerization reactions, for even such a simple polymerization as the dimerization of ferriprotoporphyrin postulated by Hogness *et al.* (67) or the dimerization of pyridine iron-protoporphyrin in alkaline solutions (15) has not yet been satisfactorily explained. In the combination of metalloporphyrins and nitrogenous compounds: $Nm + Fe \rightleftharpoons NmFe$, the value of m may vary from one to four in a single nitrogenous iron-porphyrin compound. Furthermore, when two species of nitrogenous compounds react with a metalloporphyrin there are not only substitution reactions but also addition reactions where two different species of nitrogenous compounds combine with a metal-

loporphyrin (4). To complicate matters still further, one nitrogenous compound on combining with different iron-porphyrins may or may not exhibit the polymerization phenomena.[8] Finally, data obtained from alcoholic solutions of metalloporphyrins (48, 145) must be interpreted with caution because of the possibility of combination of metalloporphyrins with alcohol (45).

The oxidation-reduction potentials of hemoglobin $\rightleftharpoons$ methemoglobin have been carefully reinvestigated by Taylor & Hastings (140). The value of $-\dfrac{\Delta E_h}{\Delta pH}$ was 0 between pH 5 and 6, curving smoothly to a line of slope -0.06 between pH 8 and 9. This behavior has led Coryell & Pauling (44) to assume the existence in methemoglobin of an acid group with a pK value of 6.65.

Our knowledge of the iron-porphyrin systems present in living cells (cytochromes, cytochrome oxidase) has advanced little since Stern's thorough review of the subject (129). Potter (122) reports that, contrary to Keilin & Hartree's findings (75), ferricytochrome-c combines with cyanide, giving as proof for this contention a shift toward the red of the absorption band at 5300 Å. The absorption spectrum of ferricytochrome-c is greatly altered on changing the hydrogen ion concentration of the solution (141). Utmost care concerning the different factors which affect the absorption spectrum must be taken on testing this observation. The extremely interesting behavior of cytochrome-c toward molecular oxygen (75), i.e., its rapid reaction in acid (pH 4 and below) and alkaline solutions (above pH 11) and sluggishness at pH values around neutrality, is a subject which deserves attention and may supply information on the nature of the relationship between electroactivity and autoxidation. As a rule, reversible systems which are electromotively sluggish (glutathione, ascorbic acid, phosphopyridine nucleotides, cytochrome-c) are not autoxidizable. Theorell & Åkesson (143) have further purified cytochrome-c by electrophoresis at pH 7.3, raising the iron content to 0.43 per cent. This lowers the calculated M.W. to 13000. Yakushiji & Okunuki (160) report the existence, in the heart, of another cytochrome, c_1, with a sharp band at 5520 Å. The reduced cytochrome-c_1 is oxidized

[8] Nicotine iron-coproporphyrin shows no polymerization, while under the same conditions nicotine iron-protoporphyrin does. According to potentiometric and spectrophotometric studies of Davies, ferroporphyrins combine with two moles of base; according to spectrophotometric studies of Zeile & Gnant (161), pyridine combines with ferroprotoporphyrin mole for mole.

by cytochrome-c. Ball & Meyerhof (7) have shown that marine animals whose blood pigment is hemocyanin have in their tissue not only the cytochrome system but also myoglobin. Here, molecular oxygen is picked up from the atmosphere and stored by copper-protein (hemocyanin), is then transferred to the tissues and used up by iron-porphyrin proteins (myoglobin, cytochromes).

The controversy regarding the role of cytochromes and cytochrome oxidase and their relationship will persist without clarification until they have been obtained in pure condition, or at least free from each other. No definite advance will be made from experiments with muscle suspensions, where all these iron-porphyrin compounds are always present. For example, Euler & Hellström (53) reported having tissue extracts from which the following fractions were separated: (i) "succinodehydrogenase"; (ii) cytochrome-c; (iii) cytochrome-b; (iv) cytochrome-a and b; and (v) cytochrome oxidase. Keilin & Hartree (76), however, maintain that Euler's preparations i, iv, and v already contained a more or less complete system.

Flavoproteins.—It becomes more and more evident that the main role of flavoproteins in biological oxidations is that of catalysts for the transfer of electrons between the sluggish oxidation-reduction systems (pyridine nucleotides) and the cytochrome system. This role was at first unrecognized, because flavoproteins, as electroactive systems, are autoxidizable. As a consequence, during the isolation of an enzyme system the cytochromes could be lost and oxidation could proceed by direct electron transfer from flavoprotein to molecular oxygen. The excellent contribution of Haas, Horecker & Hogness (64) has shown that this happened with the enzyme system for the oxidation of hexosemonophosphate, which was the first isolated by Warburg & Christian (150). It was postulated that the oxidation of hexosemonophosphate to phosphohexonic acid proceeded as follows: hexosemonophosphate $+$ Py$(PO_4)_3$-protein[4] $\rightarrow$ H_2Py$(PO_4)_3$-protein $+$ phosphohexonic acid; H_2Py$(PO_4)_3$-protein $+$ flavoprotein $\rightarrow$ Py$(PO_4)_3$ $+$ H_2 flavoprotein; H_2 flavoprotein $+$ O_2 $\rightarrow$ flavoprotein $+$ H_2O_2.

As early as 1936, Theorell (142) pointed out that the rate of oxidation of this flavoprotein at oxygen tensions existing in living tissues is so slow as to make it doubtful that this is the normal pathway of its reoxidation. Furthermore, he found that reoxidation occurred more

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[4] Py$(PO_4)_3$ = triphosphopyridine nucleotide.

rapidly by means of cytochrome-c, and suggested that the cytochrome system may be concerned in the reoxidation of the reduced flavoprotein *in vivo*. In 1939, Barron (17) pointed out that the oxidation of hexosemonophosphate by certain bacteria was completely inhibited by hydrogen cyanide, an inhibition which meant that the last link to molecular oxygen was not flavoprotein but the cytochrome system. It has now been beautifully demonstrated by Haas, Horecker & Hogness (64) that, indeed, the enzyme system concerned with the oxidation of hexosemonophosphate is made up of an activating protein, triphosphopyridine nucleotide, flavoprotein mononucleotide, and the cytochrome system. This flavoprotein oxidizes triphosphopyridine nucleotide and reduces cytochrome-c. The molecular weight of the flavoprotein is calculated as 75000. At pH 8.8 and at 0° it loses 30 per cent of its activity in two days; at pH 4.5 it is still less stable. Using the method of Warburg & Christian (150a) they were able to separate the protein from the flavin, although its affinity for the protein is much greater ($K = 1 \times 10^{-9}$ M $\times$ liter) than that of the flavin dinucleotide of amino acid oxidase ($K = 250 \times 10^{-9}$ M $\times$ liter). The old "yellow enzyme" of Warburg & Christian (150) was undoubtedly an artifact.[5] This discovery explains the findings of Hawthorne & Harrison (65a) that cytochrome-c in the presence of cytochrome oxidase, triphosphopyridine nucleotide, and the activating protein brings about the aerobic oxidation of glucose. They suggest that some factor was present in the cytochrome oxidase preparation which was responsible for the reduction of cytochrome-c. This factor was undoubtedly the flavoprotein mononucleotide of Haas, Horecker & Hogness. That the reduction of cytochrome-c during the oxidation of succinate by the preparation from muscle (70, 76) and the oxidation of lactate by yeast (49) are also carried on by a flavoprotein mononucleotide seems possible, although it has not yet been definitely established.

The role of flavoprotein dinucleotides in biological oxidations has been more extensively studied, undoubtedly because of their greater stability. Flavoprotein dinucleotide prepared from heart muscle (134) reduces diphosphopyridine nucleotide (43, 52, 108). How the reduced flavoprotein dinucleotide is oxidized in the living cell has not yet been established though the participation of cytochrome-b has been suggested (119). Abraham (1) demonstrated that the prosthetic group

[5] It is likely that Haas' "new yellow enzyme" (63), a flavin dinucleotide, transfers electrons between diphosphopyridine nucleotide and cytochrome-b.

of this flavoprotein is flavin adenine dinucleotide. Combined with the specific proteins it forms one of the components of the enzyme systems which oxidize *d*-amino acids (150a), xanthine (5, 6, 42), aldehydes (60a), and pyruvic acid (106). Although in isolated systems flavoprotein dinucleotides may be oxidized with molecular oxygen, in the aerobic living cell they must act as intermediary catalysts between the diphosphopyridine nucleotides or the protein-substrate complexes, and the cytochrome system. This assumption explains why the oxidation of *d*-amino acids and pyruvic acid by bacteria possessing cytochromes is completely inhibited by hydrogen cyanide (22, 30). When the cytochrome system is absent, as occurs in nature in some bacteria or protozoa, or by artifact in the preparation of enzyme systems, flavoprotein dinucleotide is oxidized directly by oxygen.

The discovery that flavin adenine dinucleotide catalyzes the reduction of fumarate (56, 57) makes more certain the role of enzymatic sluggish reversible systems (oxalcetate $\rightleftharpoons$ malate; succinate $\rightleftharpoons$ fumarate; α-hydroxyglutarate $\rightleftharpoons$ α-ketoglutarate) as intermediate catalysts in cell respiration.

The study of biological oxidations catalyzed by flavoproteins in cells and tissues has been difficult because there was no way of inhibiting the reaction at the point where the flavoproteins act. Krahl *et al.* (84) now report that certain phenols having nitro and halogen groups as substituents inhibit at relatively high concentrations ($0.001\ M$) the oxidation of *d*-alanine by the Warburg enzyme, amino acid oxidase. Klein & Kohn (81) report that red cells can synthesize flavin adenine dinucleotide from riboflavin *in vitro* and *in vivo*.

In Table II there is a chart summarizing the role of flavoproteins in biological oxidations. There is as yet no experimental evidence for the reaction between flavin adenine dinucleotide and cytochrome-*b*, nor for electron transfer between activated substrate and cytochrome-*c* through flavin mononucleotide.

Other electroactive systems.—A clear example of the complexity surrounding oxidation-reduction systems on combining with proteins is presented by Kuhn & Wallenfels (95) in their study of echinochromes as prosthetic groups of high molecular compounds in the eggs of *Arbacia pustulosa.* Fully matured females yielded echinochrome A; animals in the stage of early maturity had little A but large quantities of echinochrome B and C. All three echinochromes combined with proteins. They attract spermatozoa and agglutinate them; the mechanism of this reaction is still unknown, as is also the effect of

these proteins on the oxidation-reduction potential of the echino-chromes.

Although Horowitz (71) calls the yellow pigment that he has ex-tracted from the eggs of *Urechis caupo* (urechrome), a respiratory pigment, it is possible that the pigment acts like the echinochromes. Urechrome is an electroactive oxidation-reduction system (E'_o at pH $7.0 = +0.140$v.).

TABLE II

ROLE OF FLAVOPROTEINS IN BIOLOGICAL OXIDATIONS*

Substrate

d-amino acids, aldehyde $\rightarrow$ AP $\rightarrow$ FD $\nearrow^{O_2}$ Cyt. *b* ?

Succinate $\rightarrow$ AP $\rightarrow$ FM ? $\rightarrow$ Cyt. *c*

Xanthine, pyruvate, fumarate
  reduction $\rightarrow$ AP $\rightarrow$? $\rightarrow$ FD $\nearrow^{O_2}$ Cyt. *b* ?

Ethanol, phosphoglyceraldehyde, lac-
  tate, malate, glutamate, formate $\rightarrow$ AP $\rightarrow$ Py(PO$_4$)$_2$ $\rightarrow$ FD $\nearrow^{O_2}$ Cyt. *b* ?

Hexosemonophosphate, phospho-
  hexonic acid, glucose, gluta-
  mate, isocitrate $\rightarrow$ AP $\rightarrow$ Py(PO$_4$)$_3$ $\rightarrow$ FM $\nearrow^{O_2}$ Cyt. *c*

Coupled reactions between sluggish
  enzymatic reversible systems;
  Szent-Györgyi's catalysts $\rightarrow$ AP $\rightarrow$ Py(PO$_4$)$_2$H$_2$ $\rightarrow$ FD $\rightarrow$ Py(PO$_4$)$_2$

* AP, activating protein; FD, flavin adenine dinucleotide; FM, flavin adenine mono-nucleotide; Py(PO$_4$)$_2$, diphosphopyridine nucleotide; Py(PO$_4$)$_3$, triphosphopyridine nucleo-tide; Cyt. *b*, cytochrome-*b*; Cyt. *c*, cytochrome-*c*.

Sluggish reversible systems.—The oxidation-reduction potentials of diphosphopyridine nucleotides have been calculated by Ball & Ramsdell (8) by the use of flavoproteins and dyes (E'_o at pH $7.2 = -0.26$). They have also been calculated by Borsook (31) from the equilibrium measurements of the system:

$$CH_3CH_2OH + Py(PO_4)_2 \rightleftharpoons CH_3CHO + Py(PO_4)_2H_2,$$
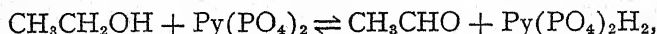
given by Negelein & Wulff (116); Borsook gives the value for the potentials as $E'_o = -0.072 - 0.03$ pH ± 0.0008, which would give an E'_o value of -0.282v. at pH 7 and 30°. This second figure seems to be more reliable.

The rapid destruction of phosphopyridine nucleotides has always been the cause of many failures of experiments carried on *in vitro*. Lennerstrand (102) reports that diphosphyridine nucleotide is rapidly inactivated by the yeast proteins of fermentation (apozymase); hexosediphosphate hinders the inactivation; activation may be partially restored by the simultaneous action of glucose, hexosediphosphate, phosphate, and adenylic acid.

It is difficult to interpret the interesting results reported by Saunders, Dorfman & Koser (127). According to them, nicotinamide added to washed suspensions of *Proteus vulgaris* (grown in synthetic media poor in phosphopyridine nucleotides) increased the oxidation of glucose twice as much as an equivalent amount of diphosphopyridine nucleotide, while it had no effect on the rate of oxidation of lactate and glutamate. Kligler & Grossowicz (82) had also spoken of nicotinic acid as activator of glucose fermentation; they have further reported that nicotinic acid amide added to *S. dysenteria* produced the complete oxidation of lactate and acetate after an incubation period. These last experiments may be interpreted as due to diphosphopyridine nucleotide synthesis. The findings of Saunders *et al.* may be due, as they state, either to permeability differences, to the formation of a new coenzyme, or simply to the catalytic action of nicotinamide ironporphyrin, which has a very high oxidation potential (Barron's unpublished experiments).

Activating Proteins[6]

Foodstuff "on collision with a specific protein molecule, the activating protein, becomes activated, i.e., a change in its electronic structure occurs, by which the electrons become ready to be transferred through the oxidation-reduction series." This definition (18) has found support in the striking experiments of Dixon & Zerfas (50). They prepared the activating protein of alcohol oxidase, according to the method of Negelein & Wulff (116), free from diphosphopyridine nucleotide. Alcohol was oxidized by this preparation with alloxan or alloxantin as the oxidizing agent; identical results were obtained with the protein of malate oxidase: malate was oxidized by alloxan. Of all the components of oxidation enzyme systems, the protein component is the only one possessing specificity in its enzymatic action, being the one that activates the oxidizable substrate.

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[6] Dehydrogenase; dehydrase; apodehydrase; *Zwischenferment*.

Work on the isolation and purification of the activating proteins is proceeding slowly. The protein for the decarboxylation of α-keto monocarboxylic acids (carboxylase), which has been isolated by Green *et al.* (61), was found to be a magnesium-diphosphothiamin-protein complex, the elements combining in a ratio of 1 : 1 : 1. The protein for the oxidation of lactate by muscle, which was isolated from heart muscle by Straub (135), was found to combine reversibly with diphosphopyridine nucleotide, and to oxidize lactate to pyruvate. The reduced pyridine nucleotide is oxidized by flavoprotein dinucleotide. The link between the reduced flavoprotein and the cytochrome system has not yet been found, although its existence is evident from the fact that the oxidation of lactate in muscle is cyanide sensitive.

The presence of sulfhydryl groups necessary for the activity of the activating proteins is becoming more and more evident. The existence of sulfhydryl groups seems to have been established for the protein of succinoxidase (69) ; of 1,3-diphosphoglyceraldehyde oxidase (59, 123), and of glycerol oxidase (19). It must, however, be stated that there is no evidence that the sulfhydryl groups act as reversible oxidationreduction systems; they seem to act only as the connecting link between enzyme and substrate.

Metalloprotein Enzyme Systems

Of the oxidation enzyme systems, the metalloprotein complex systems are simplest of all because they react directly with the oxidizable substrate, the reduced metalloprotein being oxidized by molecular oxygen.

Since Kubowitz's discovery (94) that polyphenol oxidase is a copper-protein complex there have been published a number of papers showing that other enzyme systems which catalyze the oxidation of phenolic compounds belong to this group. Laccase (78) and tyrosinase (46) are copper-protein complexes. Ascorbic acid oxidase is also a copper-protein complex (110). Enzymes belonging to this group show remarkable specificity, as shown by Nelson and his co-workers in their kinetic studies with tyrosinase (62, 120).

This same specificity is being found in studies with peroxidase. Thus, Altschul, Abrams & Hogness (2) have prepared from baker's yeast a protein-iron-protoporphyrin compound that catalyzes the oxidation of ferrocytochrome-*c* by hydrogen peroxide. The absorption spectrum of this compound, "cytochrome-*c* peroxidase," is similar to that of horse-radish peroxidase reported by Kuhn, Hand & Florkin

(96). Yet they differ in their catalytic activities, for "cytochrome-c peroxidase" is a poor catalyst for the oxidation of pyrogallol by hydrogen peroxide, and horse-radish peroxidase is a poor catalyst for the oxidation of ferrocytochrome-c by hydrogen peroxide. Catalase is an inhibitor of this reaction. Whether this protein-iron-protoporphyrin compound does normally exist in yeast or is a product of autolysis (the compound is prepared from baker's yeast subjected to twenty-four hours autolysis at 25°) remains to be determined. The same uncertainty exists in regard to its physiological role, because the distribution of catalase, the inhibitor of this enzyme, is so widespread that it is found wherever the cytochrome system is present. Furthermore, the catalase activity is greatly increased on addition of very small amounts of cytochrome-c (76). "Dihydroxymaleic acid oxidase," described by Banga & Szent-Györgyi (9) as an enzyme abundant in plants, has been identified as a peroxidase, i.e., protein-iron-protoporphyrin, by Swedin & Theorell (137).

The extreme complexity of these protein-iron-porphyrin enzyme systems is shown by Sumner's latest contribution (136) to the chemical constitution of catalase. Sumner et $al.$ have shown that catalase may exist as several different compounds similar with respect to protein in any one species, but different with respect to the number of iron-porphyrin residues. Five types of catalase are described containing different amounts of iron-protoporphyrin and a "blue substance" (produced by the opening up of the porphyrin ring with the loss of one —CH= group). The activity depends on the ratio of iron-porphyrin to "blue substance." Whether catalase undergoes reversible oxidation-reduction, as maintained by Keilin & Hartree (77) and denied by Johnson & van Schouwenburg (72), and Weiss & Weil-Malherbe (154), is still undetermined. Zeile et $al.$ (162) report the reduction of catalase with sodium hydrosulfite by previous treatment with hydrogen sulfide; reduction is obtained also in the presence of washed liver slices.

It must be mentioned that these metalloprotein enzymes act as catalysts for the oxidation of reversible oxidation-reduction systems, a catalysis which may also be performed directly by the metals in their ionic form (for example, ascorbic acid is easily oxidized by cupric ions (21).

Uricase, the enzyme system which oxidizes uric acid to allantoin, seems to be an iron-protein complex (47, 68). Whether iron is combined with the protein as ionic iron or as an iron-porphyrin has not

yet been determined; both preparations, that of Davidson and that of Holmberg, were pale brown in color, and showed a faint band of pyridine ferroporphyrin upon addition of pyridine and sodium hydrosulfite.

Carbonic anhydrase, the enzyme discovered by Meldrum & Roughton (112), is a zinc-protein complex (79) with a zinc content of 0.33 per cent. As Keilin & Mann point out, this discovery demonstrates for the first time the physiological importance of zinc, a metal which is distributed widely in living cells. The enzyme catalyzes both phases of the reversible reaction: $H_2CO_3 \rightleftharpoons CO_2 + H_2O$; is stable within wide limits of pH change; and is very sensitive to hydrogen cyanide, hydrogen sulfide, sodium azide, and to several heavy metals. Carbonic anhydrase is also inhibited by a number of oxidizing agents and is reactivated by reducing agents such as ascorbic acid and cysteine (80). Whether this inhibition and reactivation are due to the presence in the protein molecule of sulfhydryl groups which determine the activity of the enzyme has not yet been established. The certainty that carbon dioxide takes active part in cellular respiration as an oxidizing agent brings to the fore the importance of carbonic anhydrase as an enzyme which releases carbon dioxide to atmospheric oxygen and to the cell for oxidation purposes. Leiner (101) has studied its distribution in animal tissues, especially in the swim bladder of fishes.

Mann & Keilin (111) found that sulfanilamide is a specific inhibitor of carbonic anhydrase at concentrations as low as $2 \times 10^{-6} M$; the sulfonamide group is responsible for the inhibition, as sulfapyridine and sulfathiazole have no effect. This explains the respiratory disorders observed sometimes after the use of sulfanilamide as a drug.

DIPHOSPHOTHIAMIN

Much progress has been made in the study of the phosphorylated vitamin and enzyme component, thiamin. Its phosphorylation by animal tissues was demonstrated by Ochoa (117) and the mechanism of phosphorylation in yeast was extensively studied by Lipton & Elvehjem (107), and by Weil-Malherbe (152). They have independently shown that the phosphorylation is performed by phosphate transport from adenosinetriphosphate (ATP) to thiamin (T), the transport being effected only at the surface of the protein since synthesis stops as soon as there has been enough diphosphothiamin (DPT) formed to combine with the protein. The reaction appears to be reversible, with a
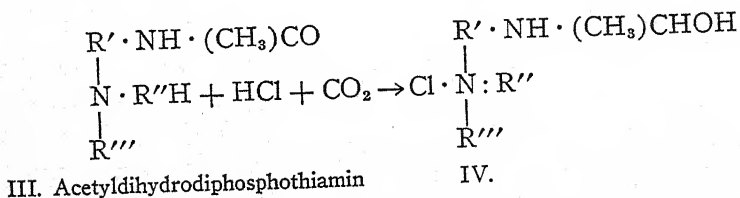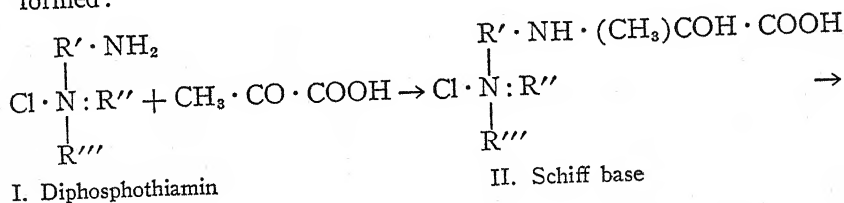
large dissociation constant, because considerable amounts of adenosine-triphosphate are required to draw the reaction toward the right side of the equilibrium point in the reaction

$$ATP + T \rightleftharpoons DPT + ADP.$$
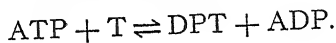
Phosphopyruvic acid could act as phosphate donator for the synthesis in presence of catalytic amounts of adenylic acid or adenosinetriphosphate.

Diphosphothiamin has already been shown to be one of the components of yeast carboxylase (109), of pyruvate oxidation (103), and of pyruvate dismutation (24). The mechanism of this catalysis is still unknown. Because thiamin contains a quaternary nitrogen, like riboflavin and the pyridine nucleotides, it has been suggested (104) that it acts as a sluggish reversible oxidation-reduction system. A great deal of work, published and unpublished, was done to prove the validity of this contention, with constantly negative results. In fact, a comparative study of the rates of reduction and reoxidation of thiamin and diphosphothiamin has shown that the vitamin becomes with phosphorylation more resistant to the action of reducing and oxidizing agents (Barron & Lyman's unpublished work).

Another interesting hypothesis has been proposed by Weil-Malherbe (153). According to him, the initial steps in the decarboxylation involve intramolecular oxidation-reduction of the Schiff base primarily formed:

$$
\begin{array}{c}
R' \cdot NH_2 \\
| \\
Cl \cdot N : R'' + CH_3 \cdot CO \cdot COOH \rightarrow Cl \cdot N : R'' \\
| \\
R'''
\end{array}
\qquad
\begin{array}{c}
R' \cdot NH \cdot (CH_3)COH \cdot COOH \\
| \\
Cl \cdot N : R'' \\
| \\
R'''
\end{array}
\rightarrow
$$

I. Diphosphothiamin   II. Schiff base

$$
\begin{array}{c}
R' \cdot NH \cdot (CH_3)CO \\
| \\
N \cdot R''H + HCl + CO_2 \rightarrow Cl \cdot N : R'' \\
| \\
R'''
\end{array}
\qquad
\begin{array}{c}
R' \cdot NH \cdot (CH_3)CHOH \\
| \\
Cl \cdot N : R'' \\
| \\
R'''
\end{array}
$$

III. Acetyldihydrodiphosphothiamin   IV.

In animal tissues, III would be oxidized to acetyl diphosphothiamin by an oxidizing catalyst (for example, flavoprotein), whereas in yeast a second intramolecular oxidation-reduction would occur resulting

in the Schiff base of acetaldehyde (IV). No evidence has been given in favor of this hypothesis, except that Langenbeck (97) from work with model decarboxylations predicted the amine nature of cocarboxylase, and showed with these models that the first step of catalysis is the formation of a Schiff base. [Schiff bases have been postulated as intermediaries in several biological reactions such as transamination and synthesis of amino acids (83, 146).] Stern & Melnick (132), on the other hand, have presented evidence against a Langenbeck cycle involving the amino groups in the pyrimidine ring. The multiple catalytic functions of diphosphothiamin (decarboxylation, oxidation, dismutation) suggested the possibility that it acts by forming the integral part of the activating protein of the enzyme systems concerned with the activation of pyruvate. The combination of diphosphothiamin-protein with pyruvate may well take place at the amino group, as suggested by Weil-Malherbe. Once the pyruvate is thus activated, it may react with catalysts for its oxidation, reduction, dismutation, or condensation. This hypothesis need not postulate a reversible oxidation-reduction of diphosphothiamin; yet it does predict more catalytic functions for the vitamin than those hitherto known. Test of this hypothesis (25, 26) has shown that diphosphothiamin accelerates in animal tissues the following reactions: (a) a carbohydrate synthesis from pyruvate, where the initial reaction seems to be: pyruvate $+$ fumarate $+ H_3PO_4 + 3 O_2 \rightarrow$ phosphopyruvate $+ 4 CO_2 + 2 H_2O$; (b) citrate synthesis from pyruvate and oxalacetate; (c) synthesis of succinate; (d) synthesis of acetoacetate. The reaction between pyruvate and glutamate to give alanine and α-ketoglutarate (transamination) was not catalyzed by diphosphothiamin (26).

Diphosphothiamin seems to be also a component of the oxidation enzyme system which in mammalian tissues oxidizes α-ketoglutarate to succinate, for the rate of its oxidation by tissues from avitaminotic animals is increased on addition of thiamin (26).

In a recent paper Krebs & Eggleston (91) suggest that diphosphothiamin is a catalyst for the reaction discovered by Wood & Werkman (158), namely the carboxylation of pyruvate to oxalacetate:

$$CH_3 \cdot CO \cdot COOH + CO_2 \rightarrow COOH \cdot CH_2 \cdot CO \cdot COOH.$$
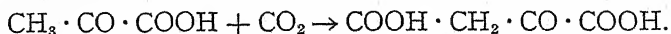
This reaction, according to the authors, would be the primary step for the synthesis of α-ketoglutarate from pyruvate, found in the liver by Evans (54). No other evidence is given for this suggestion than that previously given by other workers (since Peters' pioneer observa-

tions), the increased utilization of pyruvate by avitaminotic tissues on addition of thiamin. The authors' contention that "the vitamin takes part in a reaction of pyruvate which occurs in liver, but is of no major importance in muscle" should be quoted as a warning against rash generalizations. Thiamin had no effect "in muscle" because chopped muscle was used and phosphorylation of thiamin is almost nil under these conditions.

Of all these reactions, the decarboxylation of pyruvate to acetaldehyde and carbon dioxide and its oxidation to acetate and carbon dioxide have been most extensively studied—the decarboxylation by Stern & Melnick (130), the oxidation by Lipmann (104). Lipmann, in studying the mechanism of pyruvate oxidation, chose bacteria devoid of cytochrome, *B. Delbrückii*. According to him, there is a reversible reaction between pyruvic acid and inorganic phosphate in which pyruvic acid phosphate is formed by addition of phosphoric acid at the carbonyl group; this is oxidized to acetyl phosphate and carbon dioxide; the phosphate is transferred to adenylic acid giving acetic acid and adenosinetriphosphate. The equations were formulated by analogy to the oxidation and phosphorylation of triose phosphate studied by Warburg and his co-workers (151) and have received support by the demonstration of acetylphosphate formation (105). Lipmann found that the oxidation of pyruvate was greatly increased by addition of flavin adenine dinucleotide. The hydrogen peroxide formed during the reoxidation of the flavin by oxygen oxidized more pyruvate to acetic acid and carbon dioxide. The oxidation of pyruvate by bacteria possessing cytochrome is inhibited by hydrogen cyanide (22), an indication that the transfer of electrons from flavin to molecular oxygen is not performed directly, but through the cytochrome system. Nothing is known about the mechanism of the condensation and carboxylation reactions of pyruvate.

THE ROLE OF CARBON DIOXIDE IN CELLULAR RESPIRATION

Up to 1936, carbon dioxide reduction was considered the outstanding and exclusive characteristic of photosynthetic and chemoautotrophic organisms. However, as long ago as 1924, Warburg, Posener & Negelein (148) discovered that the rate of glycolysis in animal tissues was increased by increasing, at constant pH, the carbon dioxide pressure and bicarbonate concentration. Krebs & Henseleit (92) found that the rate of urea synthesis by mammalian liver slices increases rapidly with increasing concentrations of bicarbonate–carbon dioxide buf-

fer, and suggested (85) that the first stage in the synthesis of urea is the formation of *d*-carbamino ornithine, the carbon of the urea molecule being derived entirely from the bicarbonate of the medium [confirmed recently by Rittenberg & Waelsch (124) and Evans & Slotin (55)]. Gladstone *et al.* (60) found that *E. coli* was unable to grow when carbon dioxide was excluded from the medium.

In 1936, the role of carbon dioxide as an oxidation agent in heterotrophic cells began to be noticed. In that year, Woods (159) proved that in *E. coli* the reaction $HCOOH \rightleftharpoons H_2 + CO_2$ is a truly reversible process, carbon dioxide being reduced by molecular hydrogen to give formic acid. Barker (11) found that one species of methane-producing bacteria can carry out the reaction:

$$CO_2 + 2 C_2H_5OH \rightarrow CH_4 + 2 CH_3COOH,$$
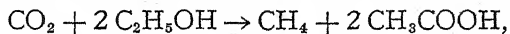
a reduction of carbon dioxide to methane, and an oxidation of ethanol to acetic acid. Wood & Werkman (156) discovered that propionic acid bacteria used up carbon dioxide during the fermentation of glycerol and postulated that carbon dioxide combined with a three-carbon compound to give succinic acid. Hamon (65) observed that under the influence of carbon dioxide, the respiration of certain yeasts increased. Finally, Wieringa (155) found that *Clostridia* are capable of oxidizing hydrogen by means of carbon dioxide, the latter being reduced to acetic acid. In 1938, Elsden (51) showed the synthesis of succinic acid from pyruvic acid and carbon dioxide, Wood & Werkman (157), the utilization of carbon dioxide by *Propionibacterium pentosaceum* during the breakdown of sugars and alcohols. In 1940, Gaffron (58) demonstrated that the oxyhydrogen reaction which occurs in certain algae adapted to molecular hydrogen when oxygen is added is considerably increased when carbon dioxide is present; carbon dioxide is absorbed and reduced, the reaction being inhibited by hydrogen cyanide. Lee & Umbreit (99) also found heterotrophic microorganisms capable of oxidizing hydrogen in the presence of carbon dioxide. The utilization of carbon dioxide in a dark reaction was thus definitely established. However, since there is production of carbon dioxide in respiration, it is clear that carbon dioxide assimilation can best be studied directly by isotopic tracer methods by the use of either stable (C^{13}) or radioactive (C^{11} and C^{14}) carbon. With this technique, the role of carbon dioxide in cellular respiration has been conclusively shown. Wood & Werkman (158) have demonstrated that propionic acid bacteria form from glycerol in the presence of carbon dioxide, propionic and succinic acid

in amounts almost equimolecular with quantities of absorbed carbon dioxide [confirmed by Carson & Ruben (35) and Carson et al. (34)]. This carbon dioxide assimilation is inhibited by fluoride and iodoacetate. For the formation of succinate from pyruvate by E. coli they postulate the previous formation of oxalacetic acid:

$$CO_2 + CH_3 \cdot CO \cdot COOH = COOH \cdot CH_2 \cdot CO \cdot COOH,$$
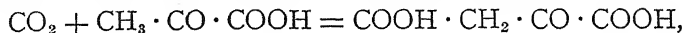
oxalacetate being reduced via fumaric acid to succinic acid.

This reaction between pyruvate and carbon dioxide occurs also in animal tissues, and according to Krebs (91) it is the primary reaction for the synthesis of α-ketoglutaric acid observed by Evans (54). Its importance is considerable, for the oxalacetate thus formed may be used by the cell for the oxidation of dihydrodiphosphopyridine nucleotide formed in the breakdown of glucose (oxidation of glycerophosphoric aldehyde to glycerophosphate). With radioactive carbon, Ruben & Kamen (125) have shown that baker's yeast, E. coli, ground plant roots, and ground liver (rat) tissues assimilate carbon dioxide, an assimilation that is inhibited by hydrogen cyanide. Barker et al. (12, 14) have demonstrated the reduction of radioactive carbon dioxide by methane-producing bacteria, and the oxidation of purines by Clostridia with carbon dioxide as the ultimate oxidizing agent. The generalized reaction would be:

$$4\,H_2\,A + 2\,CO_2 \rightarrow 4\,A + CH_3 \cdot COOH + 2\,H_2O,$$

where $H_2\,A$ is the oxidizable substrate and A, the oxidation product.

Carbon dioxide plays, therefore, the role of a highly specific oxidation agent. Almost nothing is known about the catalysts for these reactions, this being a field where intensive work needs to be done. Diphosphothiamin, according to Krebs, acts as a catalyst for the reaction $CH_3 \cdot CO \cdot COOH + CO_2 \rightarrow COOH \cdot CH_2 \cdot CO \cdot COOH$. Is this vitamin the catalyst for all reactions where CO_2 is the oxidizing agent?

VARIABILITY WITHIN SINGLE OXIDATION ENZYME SYSTEMS

It is now established that oxidation enzymes are systems made up of two main components: a specific protein, the activating protein, and a number of reversible oxidation-reduction systems which transfer electrons from oxidizable substrate to molecular oxygen. It is also established that if there is only one reversible system, it is electroactive and autoxidizable, combining readily with molecular oxygen; if there are two systems, one is sluggish, nonautoxidizable, the other electroactive. If there are more, the series of oxidation-reduction systems is

made up as follows: sluggish system $\rightleftharpoons$ electroactive system $\rightleftharpoons$ sluggish system $\rightleftharpoons$ electroactive system $\rightleftharpoons O_2$, the electrons being thus transferred by gradual steps from the system of lower oxidation potential to the system of higher oxidation potential. Evidence is also being accumulated in favor of Michaelis' theory (113) of "compulsory univalent oxidation," according to which electron transfer takes place one by one and not by pairs as customarily assumed, this univalent oxidation being performed through radical formation. In fact, radical formation has been postulated for the oxidation of the sluggish systems, phosphopyridine nucleotides (74), and for the electroactive systems, flavin (114) and flavoprotein (63), the complete path for univalent oxidation thus being possible within the enzyme system up to molecular oxygen. No evidence has yet been provided for the existence of free radical formation on oxidation of the oxidizable substrate, although the possibility of its existence is suggested from kinetic experiments (20, 64, 133). Table III shows schematically the different types of oxidation enzyme systems. All the oxidation enzyme systems discovered to date may be grouped under these six types.

TABLE III

TYPES OF OXIDATION ENZYME SYSTEMS

I.	Metalloproteins			
II.	Activating protein	Flavin		
III.	Activating protein	Flavin	Cytochrome system	
IV.	Activating protein	Flavin ?	Cytochrome system	
V.	Activating protein	Di- or tri-phosphopyridine nucleotide	Flavin	
VI.	Activating protein	Di- or tri-phosphopyridine nucleotide	Flavin	Cytochrome system

To attempt to go beyond these general postulates, i.e., to say that because an enzyme system found in a particular cell belongs to a particular type, and that oxidation of that substrate will proceed according to that type in all cells is misleading. It is time to warn against such dangerous generalizations. It is known that cells with no cytochrome system can perform a number of oxidations which require the cytochrome system in cells possessing it. The oxidation of lactate provides a good example: lactate is oxidized by cells possessing no cyto-

chrome (23), so that the lactate oxidation enzyme system here must belong either to type II or V; in yeast it seems to belong to type III because it does not require pyridine nucleotide (49); in muscle it belongs to type VI (135). Furthermore, an enzyme system may change in the same cell the number and nature of its components during its development (115). It is, in fact, well known that the metabolism of embryonic cells is different from that of the adult cell. Therefore, it is essential on investigating the mechanisms of biological oxidations and reductions to study them both comparatively and ontogenetically.

Enzymatic Sluggish Reversible Systems as Catalysts for Cellular Respiration[7]

In a series of papers published in 1935 Szent-Györgyi and his co-workers (3) suggested that in the respiration of pigeon breast muscle enzymatic reversible systems (succinate $\rightleftharpoons$ fumarate; oxalacetate $\rightleftharpoons$ malate) acted as electron mediators from the substrate to the cytochrome system. Experimental proof was offered by Stare & Baumann (128), who found that succinate, fumarate, malate, and oxalacetate acted as catalysts in the respiration of pigeon breast muscle; the increased oxygen uptake was accompanied with carbon dioxide change at a ratio similar to that found in normal respiration. According to Szent-Györgyi (138) "substrate hydrogen" is transported from dihydrodiphosphopyridine nucleotide $[(PyPO_4)_2H_2]$ to oxalacetate, which is thereby reduced to malate;[8] diphosphopyridine nucleotide is regenerated and is then available for further electron transfer. Part of the malate is oxidized by a flavoprotein and oxalacetate is regenerated; the other part is converted by fumarase to fumarate. Reduced flavoprotein is oxidized by fumarate, regenerating oxidized flavoprotein and giving succinate. Finally, succinate is oxidized by the cytochrome system and fumarate is regenerated. Thus, oxidation of $Py(PO_4)_2H_2$ is accomplished by a mechanism constantly kept in order by its automatic regeneration. In other words, a path is provided for the oxida-

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[7] This section was prepared by Dr. F. J. Stare.

[8] There seems to be confusion about the extent of these reactions, for Potter (121) has recently argued that because the system oxalacetate $\rightleftharpoons$ malate reacts reversibly with diphosphopyridine nucleotide it cannot act as electron mediator. In the reaction, $Py(PO_4)_2H_2 +$ oxalacetate $\rightleftharpoons$ malate $+ Py(PO_4)_2$, equilibrium is reached (at equal concentrations) when 99.9 per cent of $Py(PO_4)_2H_2$ is oxidized and 99.9 per cent of oxalacetate is reduced. In other words, the reaction is greatly displaced toward the right (E'_o at pH 7.0: of oxalacetate $\rightleftharpoons$ malate $= -0.102$ v.; of $Py(PO_4)_2 = -0.280$ v.).

tion of $Py(PO_4)_2H_2$ through an oxidation-reduction series made up of enzymatic sluggish reversible systems (oxalacetate $\rightleftharpoons$ malate; fumarate $\rightleftharpoons$ succinate), sluggish systems (diphosphopyridine nucleotide; cytochrome-c), and electroactive systems (flavoproteins; cytochrome oxidase?).

Krebs and his co-workers (89) have supplemented Szent-Györgyi's theory and have added citric and α-ketoglutaric acids to the cycle (the so-called citric acid cycle). According to Krebs, oxalacetate condenses with pyruvate to form an intermediate which loses carbon dioxide and hydrogen; citrate is formed. The citrate rearranges, and loses carbon dioxide and hydrogen to give α-ketoglutarate, which loses carbon dioxide and hydrogen to give succinate. Succinate is oxidized to fumarate, which is hydrated to malate. Malate is oxidized to oxalacetate, and the cycle is completed. Krebs' cycle offers a logical and picturesque pathway for the complete oxidation of carbohydrate.

Recently Krebs and his co-workers (90, 93) have reported additional observations to support the "citric acid cycle." They found (in pigeon breast muscle) that in the presence of a large concentration of malonate ($0.025\ M$), one mole of pyruvate was removed per mole of added fumarate: pyruvate $+$ fumarate $+\ 2\ O_2 =$ succinate $+\ 3\ CO_2$ $+\ H_2O$. Succinate, they say, must be formed by an oxidative process unaffected by malonate. When large concentrations of pyruvate were used ($0.03\ M$), 50 per cent yields of α-ketoglutarate and 15 per cent yields of citrate were found. The low yields of citrate were explained as due to incomplete recovery owing to the rapid removal of intermediates under the experimental conditions (an assumption that has no satisfactory proof), and to removal of some of the citrate by an anaerobic reaction with oxalacetate. An interesting schematic representation correlating the citrate cycle with the Szent-Györgyi theory in the complete oxidative breakdown of carbohydrate has been advanced by Krebs (87). The Szent-Györgyi theory is portrayed as participating in the transport of at least six of the twelve hydrogen atoms released during the oxidation of one triose equivalent via the citrate cycle. Further evidence in support of the "citric acid cycle" is given by Krebs (86) from experiments on the fate of oxalacetate when incubated anaerobically with chopped muscle. The increase in intermediates (carbon dioxide, succinate, α-ketoglutarate, fumarate, citrate) was in agreement with the "citric acid cycle" theory. Whether the succinate found was a result of reduction of oxalacetate over fumarate (158), or an oxidation of α-ketoglutarate was not determined. Again the

yields of citrate were low, being 1.2 and 2.1 per cent in the two experiments reported.

Krebs' theory has been criticized by Breusch (32) and Thomas (144), who were unable to find more than 2 per cent citrate formation from oxalacetate in muscle incubation experiments. With malonate concentrations of 0.005 M and 0.001 M the inhibition of chopped muscle respiration was prevented by the addition of appropriate concentrations of fumarate, malate, α-ketoglutarate, or succinate, but not by citrate (27). Furthermore, the increase in respiration due to citrate was always less than that due to fumarate (27, 32).

The "citric acid cycle" theory is based on certain carefully established experimental facts. Whether it represents the main pathway of the normal respiration of pigeon breast muscle has not been proved. The following reasons are offered in criticism of that hypothesis. (a) Only with high concentrations of pyruvate was Krebs able to demonstrate increases in citrate, α-ketoglutarate, and succinate, whereas pyruvate normally occurs in a relatively low concentration. Pyruvate is an extremely reactive substance, undergoes a variety of reactions (19, 129), and when present in relatively large concentrations some of it may condense with oxalacetate to give citrate and α-ketoglutarate. (b) From three independent laboratories (those of Krebs, Breusch, and Thomas) low yields of citrate following additions of pyruvate and dicarboxylic acid were obtained. (c) The anaerobic data used by Krebs (fate of oxalacetate) to supply evidence for an aerobic process, respiration, has to be accompanied by a determination of the relative rates of reactions involved in these two processes, anaerobic and aerobic. Similar evidence was offered by Krebs (88) when he postulated in earlier papers that pyruvate was utilized via dismutation. The theory was shown to be incorrect by studying the rates of pyruvate utilization aerobically and anaerobically, i.e., the oxydismutation coefficient (24). (d) An important postulate for Krebs' theory is that malonate completely prevents the anaerobic reduction of oxalacetate to succinate, but this contention is without adequate proof. In anaerobic experiments, in the absence of malonate, succinate was formed from either added α-ketoglutarate or fumarate, in fact, more so from the latter, and Krebs was unable to state the exact origin of the succinate. (e) Citrate catalysis of respiration is completely inhibited by malonate, yet normal respiration can be obtained in the presence of similar malonate concentration.

The catalytic role of enzymatic sluggish systems has been demon-

strated by two more investigators. With preparations of brain suspensions, Banga *et al.* (10) showed that the metabolism of pyruvate is greatly accelerated on addition of small amounts of fumarate. With kidney extracts Cori and his co-workers (40) have clearly demonstrated that the complete oxidation of glucose proceeds only on addition of any of these enzymatic reversible systems (isocitrate, glutamate, α-ketoglutarate, succinate).

PHOSPHORYLATIONS AND OXIDATIONS

The discussion of the oxidation enzyme systems has been limited to that of activating proteins and the oxidation-reduction systems that transfer electrons to molecular oxygen. That oxidations may proceed with only those components cannot be doubted. However, oxidations may be greatly accelerated by addition of other substances not intrinsically part of the enzyme system. Of these substances the participation of phosphorus has attracted great attention in the last two years, as well as the participation of enzymatic reversible systems in phosphorylation processes. Warburg & Christian (151) offered the first clear example of oxidation and phosphorylation. They showed that Fischer's ester (3-phosphoglyceraldehyde) reacts with phosphoric acid to give 1,3-diphosphoglyceraldehyde, which is reversibly oxidized to 1,3-diphosphoglycerate by diphosphopyridine nucleotide. The oxidation process was greatly enhanced on addition of adenylic acid, an acceleration due to the withdrawal of 1,3-diphosphoglycerate from the reversible reaction. (Adenylic acid was phosphorylated to adenosinetriphosphate, and 1,3-phosphoglycerate was dephosphorylated to 3-phosphoglycerate). The over-all reaction was oxidation of one molecule of triosephosphate coupled with the esterification of one molecule of inorganic phosphate. The reaction of the carbonyl group of pyruvate with inorganic phosphate, a compound which on oxidation yields acetyl phosphate, has been postulated by Lipmann (104). Acetyl phosphate acts then as phosphate donator over the adenylic system.

Reversible enzymatic systems also take part in these reactions. In the oxidation of pyruvate by brain extracts two atoms of phosphorus can be esterified per atom of oxygen consumed, whereas the ratio is $1:1$ when succinate is added (118). The role of succinate $\rightleftharpoons$ fumarate and other enzymatic reversible systems has been demonstrated by Belitzer & Tsibakova (28), Belitzer & Golovskaya (29), Kalckar (73), and Cori *et al.* (41). Colowick, Kalckar & Cori (40), in a beautifully conceived paper, have studied the problem quantitatively. Working

with cell-free extracts of kidney, liver, and heart muscle they have shown that these reversible systems are essential for the initial phosphorylation of glucose, the energy for the phosphorylation being provided by the oxidation of succinate. They found with heart extracts that while one molecule of glucose is completely burned, five molecules of fructose diphosphate accumulate, which corresponds to a coupling of ten of the hydrogen transfers involved in the oxidation of glucose with phosphorylation. In this simplified system the energy derived from oxidations is used only for phosphorylations. In the intact living cell the regulatory mechanisms direct the flow of energy into various channels.

ORIENTATION OF REACTIONS

Once the components of the complete oxidation enzyme systems are isolated and the mechanism of their action, when allowed to interact (in extracts and suspensions), is understood, there remains for the biologist the problem of integration. Do any of these mechanisms (found in the isolated systems, in the extracts, and in the suspensions) apply to the living cell? In oxidation-reduction systems in equilibrium, in solution, all the numerous theoretical possibilities for the integration of the energies may be integrated in what Clark calls a continuum. Such integration becomes not only difficult but sometimes misleading when attempted in dynamic, heterogenous, open systems. One example will suffice to illustrate this difficulty. The anaerobic breakdown of glucose in muscle extracts is understood, and the scheme of Embden-Meyerhof-Warburg was assumed to apply to the contracting muscle. However, Sacks (126), from experiments with the living, contracting muscle, performed with the use of radioactive phosphorus, maintains that the chemical events during muscular contraction do not follow the series of reactions detected in the extracts. Adenosinetriphosphate does not act as phosphate transporter; the phosphate necessary for the formation of hexosemonophosphate is derived directly from phosphocreatine and not from inorganic phosphate; the hexosemonophosphate formed does not undergo any further change during anaerobic contraction. The phosphate changes, according to Sacks, are not directly concerned with the formation of lactic acid. In other words, the mechanism of muscle glycolysis so carefully worked out for muscle extract is found wanting when tested in the living contracting muscle. Approaches, of course, have to be made. But attention must be constantly given to the fact that cellular respiration is the in-

tegration of a number of oxidation-reduction reactions, some of them reversible, which orient themselves in multiple manner although eventually giving the same products, carbon dioxide and water. This orientation of reactions is of primary importance for the living cell. One of them, the Pasteur Reaction, has been extensively studied. Burk (33), who has devoted to the subject two thorough and exhaustive reviews, defines it as the oxygen inhibition of fermentative processes, a definition which embraces well the complex phenomena. If only one fermentation process (glycolysis and oxidation of carbohydrate) is considered, for the sake of simplicity, the problem is to discover the mechanisms by which the formation of lactic acid is abolished in the presence of air. As Burk has demonstrated, there are several possibilities, so that no single explanation can be given for the Pasteur Reaction. Stern *et al.* (131), concentrating their attention on the phenomenon discovered by Lasser (98), namely, that at certain carbon monoxide pressures both glycolysis and respiration can be observed in the retina, have determined the absorption spectrum of what they call the "Pasteur enzyme," using the photochemical method of Warburg. They concluded that it was probably a pheohemin compound with three absorption bands with maxima at 4500 Å, 5100 Å, and 5800 Å, respectively. [Warburg & Negelein (149) from photochemical measurements in retina give also three absorption bands for the "Respiration enzyme": 4350 Å, 5460 Å, and 5780 Å.] The "Pasteur enzyme," according to Stern (129), may act "as the specific oxidant either of dihydrocozymase or of reduced thiaminpyrophosphate (coenzyme of pyruvic dehydrogenase)." No metalloporphyrin compound has, to date, been shown to react with pyridine nucleotides, and the existence of reduced thiamin pyrophosphate as coenzyme of pyruvic dehydrogenase is improbable. If Stern's postulated reaction exists, $Py(PO_4)H_2$ may be reduced through a flavoprotein by the iron-porphyrin compound.[9] Inhibition of this release mechanism would leave the enzymatic reversible systems, oxalacetate $\rightleftharpoons$ malate and pyruvate $\rightleftharpoons$ lactate,[10] which react with $Py(PO_4)_2H_2$,

[9] No cycle need be postulated for the catalytic action of enzymatic reversible oxidation-reduction systems. In fact, the regeneration of oxalacetate through reduction of diphosphopyridine nucleotide, as assumed by Szent-Györgyi, is a thermodynamically improbable reaction. Oxalacetate is regenerated by direct carboxylation of pyruvate with carbon dioxide by means of Wood & Werkman's reaction.

[10] In a recent review (18), the E'_0 for the system oxalacetate $\rightleftharpoons$ malate at pH 7.0 was given as -0.169 v. More reliable measurements made by Lehmann & Hoff-Jörgensen (100) give for E'_0 the value, -0.102 v. The reversible system

ready to compete; part of the pyruvate would be oxidized (respiration), while the other part would be reduced (glycolysis). But to postulate one pathway for the utilization of pyruvate would be to ignore the great reactivity of this compound. [See Stern's (129) and Barron's (19) tables on the sixteen different possibilities of pyruvate utilization.] Pyruvate in the living, undamaged cell may follow any of the main pathways indicated in these schemes, and the extent of the different reactions will be determined at any given time by the different factors (concentration of reactants, of electrolytes, of carbon dioxide, or of hydrogen ions; permeability factors; hormones; etc.) which at that time make up what Clark might call the cell continuum.

LITERATURE CITED

1. ABRAHAM, E. P. A., *Biochem. J.*, **33**, 543 (1939)
2. ALTSCHUL, A. M., ABRAMS, R., AND HOGNESS, T. R., *J. Biol. Chem.*, **136**, 777 (1940)
3. ANNAU, E., BANGA, I., GÖZSY, B., HUSZÁK, I., LAKI, K., STRAUB, B., AND SZENT-GYÖRGYI, A., *Z. physiol. Chem.*, **236**, 1 (1935)
4. ANSON, M. L., AND MIRSKY, A. E., *J. Gen. Physiol.*, **14**, 43 (1930–31)
5. BALL, E. G., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 100 (1939)
6. BALL, E. G., *J. Biol. Chem.*, **128**, 51 (1939)
7. BALL, E. G., AND MEYERHOF, B., *J. Biol. Chem.*, **134**, 483 (1940)
8. BALL, E. G., AND RAMSDELL, A., *J. Biol. Chem.*, **131**, 767 (1939)
9. BANGA, I., AND SZENT-GYÖRGYI, A., *Z. physiol. Chem.*, **255**, 57 (1938)
10. BANGA, I., OCHOA, S., AND PETERS, R. A., *Biochem. J.*, **33**, 1980 (1939)
11. BARKER, H. A., *Arch. Mikrobiol.*, **7**, 404, 420 (1936)
12. BARKER, H. A., *J. Biol. Chem.*, **137**, 153 (1941)
13. BARKER, H. A., RUBEN, S., AND BECK, J. V., *Proc. Natl. Acad. Sci. U.S.*, **26**, 477 (1940)
14. BARKER, H. A., RUBEN, S., AND KAMEN, M. D., *Proc. Natl. Acad. Sci. U.S.*, **26**, 426 (1940)
15. BARRON, E. S. G., *J. Biol. Chem.*, **121**, 285 (1937)
16. BARRON, E. S. G., *J. Biol. Chem.*, **133**, 51 (1940)
17. BARRON, E. S. G., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 154 (1939)
18. BARRON, E. S. G., *Physiol. Rev.*, **19**, 184 (1939)
19. BARRON, E. S. G., *Bol. soc. quím. Peru*, **6**, 7 (1940)
20. BARRON, E. S. G., AND HASTINGS, A. B., *J. Biol. Chem.*, **100**, 155 (1933)
21. BARRON, E. S. G., DeMEIO, R. H., AND KLEMPERER, F., *J. Biol. Chem.*, **112**, 625 (1935–36)

α-hydroxyglutarate $\rightleftharpoons$ α-ketoglutarate $[E'_o = -0.070$ v. (151a)] has not been considered, because its reaction with $Py(PO_4)_2H_2$ has not yet been shown to take place. The E'_o of hydroxybutyrate $\rightleftharpoons$ acetoacetate must be corrected to -0.293 v. (66).

22. BARRON, E. S. G., AND FRIEDEMANN, T. E., *J. Biol. Chem.*, 137, 593 (1941)
23. BARRON, E. S. G., AND JACOBS, H. R., *J. Bact.*, 36, 433 (1938)
24. BARRON, E. S. G., AND LYMAN, C. M., *J. Biol. Chem.*, 127, 143 (1939)
25. BARRON, E. S. G., AND LYMAN, C. M., *Science*, 92, 337 (1940)
26. BARRON, E. S. G., LYMAN, C. M., LIPTON, M. A., AND GOLDINGER, J. M., *J. Biol. Chem.* (In press)
27. BAUMAN, C. A., AND STARE, F. J., *J. Biol. Chem.*, 133, 183 (1940) .
28. BELITZER, V. A., AND TSIBAKOVA, E. T., *Biokhimiya*, 4, 516 (1939)
29. BELITZER, V. A., AND GOLOVSKAYA, K. S., *Science*, 92, 536 (1940)
30. BERNHEIM, F., BERNHEIM, M. L. C., AND WEBSTER, M. D., *J. Biol. Chem.*, 110, 165 (1935)
31. BORSOOK, H., *J. Biol. Chem.*, 133, 629 (1940)
32. BREUSCH, F. L., *Z. physiol. Chem.*, 250, 262 (1937); *Biochem. J.*, 33, 1757 (1939)
33. BURK, D., *Occasional Pub. Am. Assoc. Advancement Sci.*, 4, 121 (1937); *Cold Spring Harbor Symposia Quant. Biol.*, 7, 420 (1939)
34. CARSON, S. F., FOSTER, J. W., RUBEN, S., AND KAMEN, M. D., *Science*, 92, 433 (1940)
35. CARSON, S. F., AND RUBEN, S., *Proc. Natl. Acad. Sci. U.S.*, 26, 422 (1940)
36. CLARK, W. M., *Medicine*, 13, 207 (1934)
37. CLARK, W. M., *U.S. Pub. Health Repts.*, 38, 443 (1923)
38. CLARK, W. M., AND PERKINS, M. E., *J. Biol. Chem.*, 135, 643 (1940)
39. CLARK, W. M., TAYLOR, J. F., DAVIES, T. H., AND VESTLING, C. S., *J. Biol. Chem.*, 135, 543 (1940)
40. COLOWICK, S. P., KALCKAR, H. M., AND CORI, C. F., *J. Biol. Chem.*, 137, 343 (1941)
41. COLOWICK, S. P., WELCH, M. S., AND CORI, C. F., *J. Biol. Chem.*, 133, 359, 641 (1940)
42. CORRAN, H. S., DEWAN, J. G., GORDON, A. H., AND GREEN, D. E., *Biochem. J.*, 33, 1694 (1939)
43. CORRAN, H. S., GREEN, D. E., AND STRAUB, F. B., *Biochem. J.*, 33, 793 (1939)
44. CORYELL, C. D., AND PAULING, L., *J. Biol. Chem.*, 132, 769 (1940)
45. CORYELL, C. D., AND STITT, F., *J. Am. Chem. Soc.*, 62, 2942 (1940)
46. DALTON, H. R., AND NELSON, J. M., *J. Am. Chem. Soc.*, 60, 3085 (1938)
47. DAVIDSON, J. N., *Biochem. J.*, 32, 1386 (1938)
48. DAVIES, T. H., *J. Biol. Chem.*, 135, 597 (1940)
49. DIXON, M., AND ZERFAS, L. G., *Nature*, 143, 557 (1939)
50. DIXON, M., AND ZERFAS, L. G., *Biochem. J.*, 34, 371 (1940) .
51. ELSDEN, S. R., *Biochem. J.*, 32, 187 (1938)
52. EULER, H. V., AND ADLER, E., *Svensk Kem. Tid.*, 51, 11 (1939)
53. EULER, H. V., AND HELLSTRÖM, H., *Z. physiol. Chem.*, 260, 163 (1939)
54. EVANS, JR., E. A., *Biochem. J.*, 34, 829 (1940); *J. Biol. Chem.*, 136, 301 (1940)
55. EVANS, JR., E. A., AND SLOTIN, L., *J. Biol. Chem.*, 136, 805 (1940)
56. FISCHER, F. G., AND EYSENBACH, H., *Ann.*, 530, 99 (1937)
57. FISCHER, F. G., ROEDIG, A., AND RAUCH, K., *Naturwissenschaften*, 27, 197 (1939)

58. GAFFRON, H., *Science,* 91, 529 (1940)
59. GEMMILL, C. L., AND HELLERMAN, L., *Am. J. Physiol.,* 120, 522 (1937)
60. GLADSTONE, G. B., FILDES, P., AND RICHARDSON, G. M., *Brit. J. Exptl. Path.,* 16, 335 (1935)
60a. GORDON, A. H., GREEN, D. E., AND SUBRAHMANYAN, V., *Biochem. J.,* 34, 764 (1940)
61. GREEN, D. E., HERBERT, D., AND SUBRAHMANYAN, V., *J. Biol. Chem.,* 135, 796 (1940)
62. GREGG, D. C., AND NELSON, J. M., *J. Am. Chem. Soc.,* 62, 2500 (1940)
63. HAAS, E., *Biochem. Z.,* 298, 378 (1938)
64. HAAS, E., HORECKER, B. L., AND HOGNESS, T. R., *J. Biol. Chem.,* 136, 747 (1940)
65. HAMON, F., *Ann. physiol. physicochim. biol.,* 12, 940 (1936)
65a. HAWTHORNE, J. R., AND HARRISON, D. C., *Biochem. J.,* 33, 1573 (1939)
66. HOFF-JÖRGENSEN, E., *Skand. Arch. Physiol.,* 80, 176 (1938)
67. HOGNESS, T. R., ZSCHEILE, JR., F. P., SIDWELL, JR., A. E., AND BARRON, E. S. G., *J. Biol. Chem.,* 118, 1 (1937)
68. HOLMBERG, C. G., *Biochem. J.,* 33, 1901 (1939)
69. HOPKINS, F. G., AND MORGAN, E. J., *Biochem. J.,* 32, 611 (1938)
70. HOPKINS, F. G., LUTWAK-MANN, C., AND MORGAN, E. J., *Nature,* 143, 556 (1939)
71. HOROWITZ, N. H., *Proc. Natl. Acad. Sci. U.S.,* 26, 161 (1940)
72. JOHNSON, F. H., AND VAN SCHOUWENBURG, K. L., *Nature,* 144, 634 (1939)
73. KALCKAR, H., *Enzymologia,* 6, 209 (1939)
74. KARRER, P., AND BENZ, F., *Helv. Chim. Acta,* 19, 1028 (1936)
75. KEILIN, D., AND HARTREE, E. F., *Proc. Roy. Soc. (London), B,* 122, 298 (1937)
76. KEILIN, D., AND HARTREE, E. F., *Proc. Roy. Soc. (London), B,* 129, 277 (1940)
77. KEILIN, D., AND HARTREE, E. F., *Proc. Roy. Soc. (London), B,* 124, 397 (1938)
78. KEILIN, D., AND MANN, T., *Nature,* 143, 23 (1939)
79. KEILIN, D., AND MANN, T., *Biochem. J.,* 34, 1163 (1940)
80. KIESE, M., AND HASTINGS, A. B., *J. Biol. Chem.,* 132, 268, 281 (1940)
81. KLEIN, J. R., AND KOHN, H. I., *J. Biol. Chem.,* 136, 177 (1940)
82. KLIGLER, I. J., AND GROSSOWICZ, N., *J. Bact.,* 38, 309 (1939); *Nature,* 146, 652 (1940)
83. KNOOP, F., *Z. physiol. Chem.,* 67, 481 (1910)
84. KRAHL, M. E., KELTCH, A. K., AND CLOWES, G. H. A., *J. Biol. Chem.,* 136, 563 (1940)
85. KREBS, H. A., *Ergeb. Enzymforsch.,* 3, 247 (1934)
86. KREBS, H. A., *Biochem. J.,* 34, 460 (1940)
87. KREBS, H. A., *Biochem. J.,* 34, 775 (1940)
88. KREBS, H. A., AND JOHNSON, W. A., *Biochem. J.,* 31, 772 (1937)
89. KREBS, H. A., AND JOHNSON, W. A., *Enzymologia,* 4, 148 (1937)
90. KREBS, H. A., AND EGGLESTON, L. V., *Biochem. J.,* 34, 442 (1940)
91. KREBS, H. A., AND EGGLESTON, L. V., *Biochem. J.,* 34, 1383 (1940)
92. KREBS, H. A., AND HENSELEIT, K., *Z. physiol. Chem.,* 210, 33 (1932)

93. Krebs, H. A., Eggleston, L. V., Kleinzeller, A., and Smyth, D. H., *Biochem. J.*, **34**, 1234 (1940)
94. Kubowitz, F., *Biochem. Z.*, **292**, 221; **293**, 308 (1937)
95. Kuhn, R., and Wallenfels, K., *Ber.*, **72**, 1407 (1939); **73**, 458 (1940)
96. Kuhn, R., Hand, D. B., and Florkin, M., *Z. physiol. Chem.*, **201**, 255 (1931)
97. Langenbeck, W., *Ergeb. Enzymforsch.*, **2**, 314 (1933)
98. Lasser, H., *Biochem. J.*, **31**, 1677 (1937)
99. Lee, S. B., and Umbreit, W. W., *Zentr. Bakt. Parasitenk.*, II, **101**, 354 (1940)
100. Lehmann, J., and Hoff-Jörgensen, E., *Skand. Arch. Physiol.*, **82**, 113 (1937)
101. Leiner, M., *Naturwissenschaften*, **28**, 165 (1940)
102. Lennerstrand, Å., *Arkiv Kemi Mineral. Geol., B*, **14**, No. 1 (1940)
103. Lipmann, F., *Nature*, **140**, 25 (1937)
104. Lipmann, F., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 2218 (1939)
105. Lipmann, F., *J. Biol. Chem.*, **134**, 463 (1940)
106. Lipmann, F., *Nature*, **143**, 436 (1939)
107. Lipton, M. A., and Elvehjem, C. A., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 184 (1939); *Nature*, **145**, 227 (1940); *J. Biol. Chem.*, **136**, 637 (1940)
108. Lockhart, E. E., *Biochem. J.*, **33**, 613 (1939)
109. Lohmann, K., and Schuster, P., *Biochem. Z.*, **294**, 188 (1937)
110. Lovett-Janison, P. L., and Nelson, J. M., *J. Am. Chem. Soc.*, **62**, 1409 (1940)
111. Mann, T., and Keilin, D., *Nature*, **146**, 164 (1940)
112. Meldrum, N. U., and Roughton, F. J. W., *J. Physiol.*, **80**, 113, 143 (1933)
113. Michaelis, L., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 33 (1939)
114. Michaelis, L., Schubert, M., and Smythe, C. V., *J. Biol. Chem.*, **116**, 587 (1936)
115. Needham, J., and Nowinski, W. W., *Biochem. J.*, **31**, 1165 (1937)
116. Negelein, E., and Wulff, H.-J., *Biochem. Z.*, **289**, 436; **293**, 351 (1937)
117. Ochoa, S., *Biochem. J.*, **33**, 1269 (1939)
118. Ochoa, S., *Nature*, **146**, 267 (1940)
119. Okunuki, K., and Yakushiji, E., *Proc. Imp. Acad. (Tokyo)*, **16**, 144 (1940)
120. Parkinson, G. G., and Nelson, J. M., *J. Am. Chem. Soc.*, **62**, 2500 (1940)
121. Potter, V. R., *Medicine*, **19**, 441 (1940)
122. Potter, V. R., *J. Biol. Chem.*, **137**, 13 (1941)
123. Rapkine, L., *Biochem. J.*, **32**, 1729 (1938)
124. Rittenberg, D., and Waelsch, H., *J. Biol. Chem.*, **136**, 799 (1940)
125. Ruben, S., and Kamen, M. D., *Proc. Natl. Acad. Sci. U.S.*, **26**, 418 (1940)
126. Sacks, J., *Am. J. Physiol.*, **105**, 151 (1938); **129**, 227 (1940)
127. Saunders, F., Dorfman, A., and Koser, S. A., *J. Biol. Chem.* (In press)
128. Stare, F. J., and Baumann, C. A., *Proc. Roy. Soc. (London), B*, **121**, 338 (1936)
129. Stern, K. G., *Ann. Rev. Biochem.*, **9**, 1 (1940)

130. Stern, K. G., and Melnick, J. L., *Enzymologia,* **8,** 129 (1940)
131. Stern, K. G., Melnick, J. L., and DuBois, D., *Science,* **91,** 2366 (1940)
132. Stern, K. G., and Melnick, J. L., *J. Biol. Chem.,* **131,** 597 (1939)
133. Stotz, E., and Hastings, A. B., *J. Biol. Chem.,* **118,** 479 (1937)
134. Straub, F. B., *Biochem. J.,* **33,** 787 (1939)
135. Straub, F. B., *Biochem. J.,* **34,** 483 (1940)
136. Sumner, J. B., Dounce, A. L., and Frampton, V. L., *J. Biol. Chem.,* **136,** 343 (1940)
137. Swedin, B., and Theorell, H., *Nature,* **145,** 71 (1940)
138. Szent-Györgyi, A., *Studies on Biological Oxidation and Some of Its Catalysts* (Leipzig, 1937)
139. Taylor, J. F., *J. Biol. Chem.,* **135,** 569 (1940)
140. Taylor, J. F., and Hastings, A. B., *J. Biol. Chem.,* **131,** 649 (1939)
141. Theorell, H., *Biochem. Z.,* **285,** 207 (1936)
142. Theorell, H., *Biochem. Z.,* **288,** 317 (1936); *Nature,* **138,** 687 (1936)
143. Theorell, H., and Åkesson, Å., *Science,* **90,** 67 (1939)
144. Thomas, Q., *Enzymologia,* **7,** 231 (1937)
145. Vestling, C. S., *J. Biol. Chem.,* **135,** 623 (1940)
146. Vigneaud, V. du, Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.,* **131,** 273 (1939)
147. Warburg, O., *Angew. Chem.,* **45,** 1 (1932)
148. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.,* **152,** 309 (1924)
149. Warburg, O., and Negelein, E., *Biochem. Z.,* **214,** 101 (1929)
150. Warburg, O., and Christian, W., *Biochem. Z.,* **254,** 438 (1932); **266,** 377 (1933)
150a. Warburg, O., and Christian, W., *Biochem. Z.,* **298,** 150 (1938)
151. Warburg, O., and Christian, W., *Biochem. Z.,* **303,** 40 (1939)
151a. Weil-Malherbe, H., *Biochem. J.,* **31,** 2080 (1937)
152. Weil-Malherbe, H., *Biochem. J.,* **33,** 1996 (1939)
153. Weil-Malherbe, H., *Nature,* **145,** 106 (1940)
154. Weiss, J., and Weil-Malherbe, H., *Nature,* **144,** 866 (1939)
155. Wieringa, K. T., *Antonie van Leeuwenhoek,* **3,** 1 (1936)
156. Wood, H. G., and Werkman, C. H., *Biochem. J.,* **30,** 48 (1936)
157. Wood, H. G., and Werkman, C. H., *Biochem. J.,* **32,** 1262 (1938); **34,** 7, 129 (1940)
158. Wood, H. G., and Werkman, C. H., *J. Biol. Chem.,* **135,** 789 (1940)
159. Woods, D. D., *Biochem. J.,* **30,** 515 (1936)
160. Yakushiji, E., and Okunuki, K., *Proc. Imp. Acad. (Tokyo),* **16,** 299 (1940)
161. Zeile, K., and Gnant, G., *Z. physiol. Chem.,* **263,** 147 (1940)
162. Zeile, K., Fawaz, G., and Ellis, V., *Z. physiol. Chem.,* **263,** 181 (1940)

PROTEOLYTIC ENZYMES

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During the past year, publications on proteolytic enzymes have, in general, continued along lines of research indicated in previous reviews (1, 2). Developments of particular interest have been studies on the fractionation of crystalline pepsin, the isolation of a yeast peptidase in a highly purified form, studies on the specificity of crystalline carboxypeptidase, and a series of papers on the stereochemical specificity of proteolytic enzymes in sera and tissues of cancerous patients and experimental animals. The last mentioned contributions represent an effort on the part of several workers to ascertain whether there exists a correlation between tumor growth and changes in the specificity of the proteolytic enzymes. At present, the experimental data on the proteolytic enzymes of cancerous sera and tissues are still under discussion.

PURIFICATION AND PROPERTIES

Pepsin.—Although swine pepsin was the first of the proteinases to be crystallized, it has been clear for several years that the usual crystalline preparations do not represent a homogeneous material. Herriott, Desreux & Northrop (3) have reported the preparation of several active protein components from crystalline swine pepsin. These authors have obtained a pure "A" pepsin of constant solubility from pepsinogen and from certain commercial pepsin preparations. It has proved impossible to separate the "A" pepsin from the other protein components by means of electrophoresis at pH 4.1 to 5.2. The specific activity per milligram of protein nitrogen did not change, thus indicating that the protein components apparently migrate at the same rate in the electric field. The results of Ågren & Hammarsten (4) and Tiselius, Henschen & Svensson (5) showing an increase in specific activity per milligram of total nitrogen upon electrophoresis have been explained on the basis that the nonprotein nitrogen migrates at a different rate from that of the protein nitrogen.

Herriott, Desreux & Northrop have confirmed the electrophoresis data of Tiselius, Henschen & Svensson on the isoelectric point of purified pepsin. They have found that solutions of pure "A" pepsin, when

freed of nonprotein nitrogen, are negatively charged even in 0.1 N HCl.

Herriott, Desreux & Northrop have reported extensive studies (6, 7) on the solubility of crystalline swine pepsin. The solubility curves show, in agreement with previous studies, that more than one protein is present in crude preparations. One of these proteins has been isolated by repeated extraction with 0.6 saturated magnesium sulfate at pH 5.0. This more soluble material has been crystallized and has constant activity $(0.33[\text{P.U.}]^{\text{Hb}}_{\text{P.N.}})$[1] and constant solubility. A more insoluble fraction has been prepared by repeated precipitation with 0.45 saturated magnesium sulfate at pH 5.0. This fraction has about two-thirds the activity of the above soluble fraction as measured by the hemoglobin method $(0.2[\text{P.U.}]^{\text{Hb}}_{\text{P.N.}})$, but the same or slightly higher activity as measured by milk clotting, digestion of casein, or changes in the viscosity of casein or gelatin. This second fraction does not have constant solubility. The authors discuss the possibility that the insoluble fraction is a decomposition product of the more soluble fraction. It was also found possible to obtain from pepsinogen a pepsin fraction that has nearly twice as much enzymatic activity on hemoglobin as the above soluble fraction. The authors point out that the inhomogeneity of swine pepsin preparations may be due to the fact that the enzyme material is usually obtained from a number of different individuals of different ages, varieties, and physiological condition.

Norris & Elam (8) have described in detail the preparation from salmon stomachs of a crystalline protein that acts on denatured hemoglobin at pH 1 to 4. Although it has been designated a "pepsin" because of its acid pH optimum, the protein differs in its crystalline form (needles) and other properties from the crystalline swine and beef pepsins of Northrop. Solubility data tend to indicate that both the amorphous and twice crystallized preparations contain only one protein. On analysis, 6.7 per cent tyrosine, 1.2 per cent tryptophane, and 2.0 per cent cystine were found. The authors employed the Anson hemoglobin method for the estimation of enzyme activity; they found that addition of 0.1 N NaCl markedly increased the rate of hydrolysis while addition of cysteine or iodoacetic acid had little effect at pH 1.2.

Matthews, Dow & Anderson (9) have studied the effects of high pressure on the activity of pepsin.

Trypsin.—Horwitt (10) has shown that heparin inhibits the pro-

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[1] The term $[\text{P.U.}]^{\text{Hb}}_{\text{P.N.}}$ is used by the authors to designate pepsin units per mg. protein nitrogen with hemoglobin as the substrate.

teolytic activity of crystalline trypsin on casein as measured by the formol titration. The activity of chymotrypsin is not affected by heparin. The author has related the antitryptic activity of heparin to its anticoagulant action. Glazko & Ferguson (11) have confirmed this finding and have also shown that heparin inhibits the action of trypsin on thrombin preparations. These authors attribute the progressive inactivation of thrombin principally to the hydrolytic action of the "serum tryptase" usually present in thrombin preparations (12).

Chymotrypsinogen.—Butler (13) has attempted to fractionate crystalline chymotrypsinogen by means of magnesium sulfate and has shown that a small amount of impurity (less than 5 per cent) is concentrated in the fractions first precipitated. This impurity affects the solubility but is insufficient to influence the other properties of the material. The solubility of the final fraction is independent of the amount of saturating solid in solvents of pH 4, 5, and 8. The solubility in approximately 0.4 saturated magnesium sulfate is independent of the concentration of magnesium sulfate in the solution in which the crystals are formed and the same equilibrium is attained from supersaturated as from undersaturated solutions. It is concluded therefore that this purified chymotrypsinogen conforms closely with the phase rule criteria of a pure protein.

Cathepsin.—Anson has revised his previous procedure (14) for the extraction of the "proteinase" of animal tissues. In a recent paper (15) a method is described in which beef spleen is autolyzed and some inert material is removed by adsorption on the spleen material. Additional inert material is removed by means of aluminum hydroxide. The enzyme could be precipitated from dilute solution by means of tungstic acid and extracted from the precipitate by means of barium hydroxide–barium chloride. The material thus obtained is reported to be inactive toward gelatin at pH 3.5 in the presence of cysteine.

Freudenberg (16) has reported the presence of cathepsin in the stomach juice of humans.

Plant proteinases.—Winnick, Davis & Greenberg (17) have studied the proteolytic system of the milkweed, *Asclepias speciosa Torr.* The enzyme, named asclepain *s* (18), behaves similarly to papain in its activation by sulfhydryl compounds. The authors have performed studies on the kinetics of protein digestion (as measured by the Anson hemoglobin method) and heat inactivation of the crude enzyme.

In a later communication Greenberg & Winnick (18) reported the preparation of plant proteinases from the milkweed, *Asclepias mexi-*

cana, and from the horse nettle, *Solanum elaeagnifolium.* These enzymes have been named asclepain *m* and solanain respectively. The above authors have studied the pH optimum of the action of various plant proteinases on denatured hemoglobin. Bromelin shows a rather flat optimum region from pH 5 to pH 8. Asclepain *m* and *s* show optima near pH 7.5. Solanain has no action below pH 6 and acts optimally near pH 8.5. With urea-denatured egg albumin as the substrate the pH optima were as follows: asclepain *m,* asclepain *s,* papain, and solanain—pH 7.5; bromelin—pH 6.5 to 8.5.

Balls, Thompson & Jones (19) have studied the inhibition of coagulation of papaya latex by fluoride, citrate, and oxalate. They observed that enzymatically active press juices could be obtained from leaves, stalks, flower stems, and bark of *Carica papaya.* Balls, Lineweaver & Schwimmer (20) have found that papain is rapidly inactivated during, and soon after, the drying of the papaya latex. The inactivation occurs more rapidly in air than *in vacuo.* A significant portion of the enzyme activity is irreversibly destroyed in this process. In dilute solutions, crude papain is inactivated very rapidly while crystalline papain is apparently fairly stable.

Dyckerhoff & Gigante (21) have found that when papain solutions are heated from 50° to 70° C., the ability to convert fibrinogen to fibrin is lost while the proteolytic activity toward gelatin is retained. On heating dry papain to 100° C., the proteolytic activity disappears without loss of the thrombin action. The authors conclude that papain preparations contain a phytothrombin distinct from the proteolytic agent.

Haas (22) has reported that repeated injection of a solution of partially purified papain into guinea pigs resulted in sensitization of the animals to subsequent injections. In rabbits, injection of papain caused the formation of specific antibodies that could be demonstrated by means of complement fixation and precipitin reactions. On addition of immune serum to papain, the enzymatic activity on gelatin or casein was greatly inhibited.

Rennin.—Schöberl & Rambacher (23) have reported experiments on the purification of rennin by means of chromatographic adsorption on aluminum oxide at pH 4.2 and pH 5.6. The enzyme was concentrated in relatively narrow bands in the adsorption column and was eluted by means of phosphate buffer. The eluted enzyme is readily destroyed by papain and is not activated by the addition of reduced glutathione.

Peptidases.—Johnson (24) has reported the isolation, from auto-lyzed brewer's yeast, of a protein that hydrolyzes tripeptides (*l*-leucyl-glycylglycine, *l*-alanylglycylglycine) and dipeptides (*l*-leucylglycine, *l*-alanylglycine). Various physical methods (sedimentation, electro-phoresis, diffusion) indicate the protein to be essentially homogeneous. The protein is insoluble in water at pH 4.5 to 5.2 but is soluble in dilute salt solutions at all pH values. The enzymatic activity is lost at pH values below 4.5. A striking property of this material is the fact that zinc ions and chloride ions are essential for enzymatic ac-tivity.

Berger & Johnson (25) have continued their studies on leucyl peptidase (26) and report the finding of this enzyme in extracts of human duodenum, rat intestine, rat carcinoma tissue, chick intestine, trout and lobster tissues, and several species of bacteria. The enzyme could not be found in brewer's yeast or molds.

Maschmann (27) has pursued his studies on the bacterial pep-tidases and has reported a fractionation of the peptidases from cul-tures of *B. histolyticus* and *B. botulinus* by means of ammonium sulfate. This group of enzymes have been designated anaeropeptidases and require cysteine and ferrous ions for full activity (2). The activity toward glycylglycylglycine is precipitated by 65 per cent ammonium sulfate while the activity toward leucylglycylglycine is precipitated by 75 per cent ammonium sulfate. In another paper (28), Maschmann reports that the hydrolysis of dipeptides by the anaerodipeptidases is not inhibited by hydrazine, phenylhydrazine, hydroxylamine, or semi-carbazide and therefore concludes that a carbonyl group essential for activity is not present in the enzyme. Additional papers on peptidases have been published by Frankenthal (29) and Dale (30).

SPECIFICITY

Pepsin.—An extremely interesting study of the sedimentation rate and electrophoretic mobility of the peptic digestion products of the action of crystalline pepsin on crystalline egg albumin has been re-ported by Tiselius & Eriksson-Quensel (31). Their results tend to indicate that pepsin attacks only a few substrate molecules in a given time interval and that these are rapidly disintegrated into small par-ticles of molecular weight *ca.* 1,000. It was not possible to demonstrate the presence of any protein digestion product of a molecular weight between 1,000 and 40,000.

It had previously been shown by Fruton & Bergmann (32) that

peptides of simple structure such as glycyl-*l*-glutamyl-*l*-tyrosine are hydrolyzed by crystalline swine pepsin. Tazawa (33) has suggested that the splitting of these substrates is due to the presence of a specific polypeptidase present as an impurity. According to Tazawa the substrate for the "genuine proteinase" in pepsin is histidine anhydride. The pepsin prepared by Tazawa differs from that of Northrop and is probably less pure. Tazawa's claim has not been substantiated in the laboratory of the reviewers (34) who found that histidine anhydride is quite resistant to the action of crystalline swine pepsin (Northrop) at pH 1 to 3. It will be recalled that during recent years there has been a Japanese group that has claimed that diketopiperazines of basic amino acids are hydrolyzed by pepsin while diketopiperazines of acidic amino acids are split by trypsin and papain. In the case of the two latter proteinases, several investigators have failed to repeat the experiments of the Japanese group; more recently, Abderhalden (35) has reported that glycyl-*l*-glutamic acid anhydride is not split by pancreatin.

Fruton & Bergmann (36) have reported that crystalline salmon pepsin does not hydrolyze the typical simple substrates of crystalline swine pepsin and therefore conclude that the two enzymes differ in specificity.

Uchino (37) and Nakamura (38) have studied the relative rates of digestibility of various proteins by crude pepsin, partially purified pancreatin, and papain.

Carboxypeptidase.—A systematic study of the specificity of crystalline carboxypeptidase has been performed by Hofmann & Bergmann (39). A striking finding was the fact that the amount of enzyme required for a given hydrolytic action varies greatly with the structure of the substrate. For example, for an appreciable hydrolysis of carbobenzoxyglycyl-*l*-alanine there is required approximately 2,000 times as much enzyme as is needed for the hydrolysis of carbobenzoxyglycyl-*l*-phenylalanine. The latter compound is suggested as a desirable substrate for determination of carboxypeptidase activity. The hydrolysis of carbobenzoxyglycyl-*l*-phenylalanine follows the kinetics of a first-order reaction and the rate constant is proportional to the enzyme concentration. In the previous literature, chloroacetyl-*l*-tyrosine had been employed as the standard substrate for carboxypeptidase. The use of this compound is accompanied by at least two important disadvantages: during the hydrolysis, free tyrosine crystallizes out, and, what is more important, the enzyme is inhibited by the chloroacetic acid liberated during the hydrolysis. Consequently, first-order

kinetics cannot be obtained. It was noted that formaldehyde also inactivates carboxypeptidase at 25° and 40°.

A few years ago Abderhalden & Abderhalden (40) claimed that the hydrolysis of acylated amino acids such as chloroacetyltyrosine or chloroacetyltryptophane was due to an "acylase" different from the "genuine" carboxypeptidase which was supposed to hydrolyze acyl peptides. According to these authors, the carboxypeptidase action was observed to be inactivated by heat much more rapidly than was the acylase action. Hofmann & Bergmann have shown that this conclusion is erroneous since in the experiments of Abderhalden & Abderhalden allowance was not made for the fact that the amount of enzyme required by the different substrates varies widely. Hofmann & Bergmann have found that when this variation is taken into account, the activity of crystalline carboxypeptidase at 40° toward chloroacetyl-l-tryptophane and carbobenzoxyglycyl-l-alanine disappears in a parallel manner. These results give no indication for the enzymatic inhomogeneity of crystalline carboxypeptidase.

Additional papers on the specificity of peptidases have been published by Utzino & Nakayama (41) and Kazama (42, 43).

Activation of Proteolytic Enzymes

Fruton & Bergmann (44) have found that on precipitation of cyanide-papain by means of isopropyl alcohol, the precipitate is inactive toward several synthetic substrates. On addition of fresh cyanide, the original activity is restored. This result has been considered to be incompatible with the oxidation-reduction theory of papain activation and has been interpreted as indicating that the activator combines with the enzyme to give a reversibly dissociable addition compound. Greenberg & Winnick (18) have suggested that in the above experiment the inactivation was caused by air oxidation. In more recent experiments, Irving, Fruton & Bergmann (45) have inactivated cyanide-papain by removal of hydrogen cyanide *in vacuo*.

Greenberg & Winnick report that asclepain m and bromelin are activated by cysteine, cyanide, and sulfide (substrate—denatured hemoglobin). The authors claim to have completely inactivated asclepain m by means of hydrogen peroxide and then restored 90 to 100 per cent of the full activity by the addition of cyanide or cysteine. To the knowledge of the reviewers this is the first example of such complete reactivation of a papainase oxidized by hydrogen peroxide. Greenberg & Winnick also report that they have been unable to re-

peat the experiment of Maschmann (46) who found that papain inactivated by iodoacetic acid can be reactivated by alcohol precipitation.

Solanain differs from the other plant proteinases in its activation behavior. Its activity is not affected by cysteine, cyanide, or hydrogen sulfide and it is not inactivated by hydrogen peroxide, iodoacetic acid, etc.

Schöberl (47) has reviewed the data on the presence of sulfhydryl groups in papain and other proteinases in the light of the activation properties of these enzymes.

Berger & Johnson (48) have found that cysteine in the presence of manganous ion activates the splitting of dl-leucylglycine by yeast autolysates, swine intestinal extracts, chick intestinal tract, and B. megatherium. In contrast, the hydrolysis of dl-alanylglycine is inhibited by cysteine, plus manganous ion. It is significant that these authors found an appreciable hydrolysis of d-leucylglycine by yeast or swine intestinal extracts in the presence of cysteine and manganous ion.

Talce-Niedra (49) has reported on the activation of bacterial enzymes that cause milk coagulation.

Synthesis by Proteolytic Enzymes

The discussion regarding the reputed synthesis of protein by aeration of proteinase digests has been continued by Linderstrøm-Lang & Johansen (50) who reiterate their failure to repeat the positive results of Maver & Voegtlin (51) with papain digests of fibrin.

Strain (52) has reported that concentration of yeast autolysates does not lead to synthesis of protein. Oxygenation also fails to increase the protein content. This author calls attention to the fact that reactions between sugars and amino compounds may complicate interpretation of reputed syntheses by proteolytic enzymes. In this connection it is of interest that Ågren (53) has reported that the disappearance of amino nitrogen in certain liver extracts (54) involves the interaction between amino groups and sugars and that this reaction is catalyzed by phosphate.

Strain & Linderstrøm-Lang (55) have studied the behavior of concentrated papain digests of wool on exposure to air. A decrease of the nitrogen soluble in trichloroacetic acid is observed but this decrease is not accompanied by a corresponding drop in amino and carboxyl groups. There is therefore no indication for the synthesis of peptide bonds. However, all the sulfhydryl groups disappear in air

and the authors suggest that the change in solubility is due to the formation of disulfide linkages between peptide chains.

Collier (56, 57, 58) has studied the formation of plasteins by papain and crystalline pepsin. He found that at pH 4.8 and in the presence of an activator such as cyanide, cysteine, or hydrogen sulfide, papain is capable of forming plasteins from concentrated peptic or papain digests of egg albumin. The plastein is hydrolyzed by activated papain optimally at pH 4.2. Thus the concentration of the split products alone determines the direction of enzyme action, since the optimum pH and need for activator are nearly identical for the formation and hydrolysis of plastein.

In plastein formation from peptic egg albumin digests by crystalline pepsin, free amino and carboxyl groups and "reactive tyrosine" are decreased. Enzymatic hydrolysis of the plastein formed results in the liberation of these groups. As evidence of the protein nature of the peptic plastein, experiments are presented to show that a solution of this material in urea gives a slight anaphylactic reaction in guinea pigs. Furthermore, analysis of the material (in urea solution) in the velocity centrifuge indicates that particles of protein dimensions are present, but the sedimentation occurs in an inhomogeneous manner. The original peptic digest shows no sedimentation whatever.

The antipodal selectivity of papain in performing synthetic reactions has been employed to prepare *d*-glutamic acid from *dl*-glutamic acid by enzymatic resolution (59). Carbobenzoxy-*dl*-glutamic acid reacts with aniline in the presence of cysteine-papain to give the insoluble carbobenzoxy-*l*-glutamic acid anilide. The remaining carbobenzoxy-*d*-glutamic acid is hydrogenated to yield the desired *d*-amino acid. This method should be of value in resolving other *dl*-amino acids and peptides.

Additional papers on synthesis by proteolytic enzymes have been published by Chen (60).

Proteolytic Enzymes and Cancer

During the past year considerable work on the proteolytic enzymes of animals with tumors has been reported; however, most of the experiments have been of a preliminary nature and no clear picture has emerged as yet. Waldschmidt-Leitz & Mayer (61) have reported that while normal sera hydrolyze *dl*-leucylglycine to 50 per cent, sera of patients with cancer hydrolyze the *d*-component of *dl*-leucylglycine also, resulting in a total hydrolysis of as much as 100 per cent. Un-

fortunately, the data were calculated erroneously and a recalculation would indicate that the normal sera are also capable of attacking *d*-leucylglycine. Bayerle & Podloucky (62) have performed extensive experiments with normal and cancerous sera and have not observed a splitting of *dl*-leucylglycine that exceeded 50 per cent. In a later paper (63) these authors report that *d*-leucylglycine and *d*-leucylglycylglycine are not hydrolyzed either by normal or by cancerous sera. Bayerle & Podloucky also failed to repeat experiments of Waldschmidt-Leitz, Mayer & Hatschek (64) in which the injection into mice of a racemic peptide (*dl*-glutamylglycine) inhibited the development of carcinoma produced by benzpyrene. In a reply to these negative findings, Waldschmidt-Leitz & Hatschek (65) fail to resolve the difficulty. Euler & Skarzynski (66, 67) have reported that sera of rabbits with Brown-Pearce carcinoma or of rats with Jensen sarcoma fail to split *d*-leucylglycine and hydrolyze *dl*-alanylglycine only to 50 per cent. Several human sera of normal and cancerous subjects did hydrolyze *d*-leucylglycine after long incubation periods. The authors discuss the possibility that a specific bacterial infection may be responsible for the hydrolysis of the *d*-peptide. Abderhalden & Abderhalden (68) have also reported that nine out of forty-one sera of patients with cancer were found to hydrolyze slowly the peptides *d*-leucylglycylglycine and *d*-alanylglycylglycine. Forty-eight out of forty-nine normal sera showed no hydrolysis of these *d*-peptides. Herken & Erxleben (69) have introduced a *d*-amino acid oxidase method for the estimation of the hydrolysis of *d*-peptides. According to these authors several sera from patients with tumors showed a hydrolysis of *d*-leucylglycylglycine to 2.5 per cent after prolonged incubation. At the present writing, the status of this problem is dubious.

Fruton, Irving & Bergmann (70) have subjected a variety of simple substrates for known proteinases to the action of partially purified extracts of tumors and, in some cases, have observed a hydrolysis of peptides containing *d*-amino acids. Since a majority of the tumors tested showed no action toward these *d*-peptides, no generalization regarding an abnormal stereochemical specificity of tumor extracts could be drawn. Abderhalden & Caesar (71) have reported that liver and kidney press juices are capable of hydrolyzing both optical isomers of glycylnorleucine and glycylisoleucine. Press juices from carcinomatous liver tissues act in the same manner as those from normal livers.

Bayerle (72) has subjected liver metastases to the successive action of autolytic enzymes, pepsin, pancreatin, and erepsin. Nearly all the

potential amino nitrogen (as determined after acid hydrolysis) was liberated in this procedure. The author discusses these results in the light of Kögl's views. In a later paper (73) the author reports that destruction of the autolytic enzymes of tumor tissues by heat does not alter the sensitivity of the proteins of such tissues to the attack of pepsin or pancreatin.

A general discussion of the role of proteolytic enzymes in tumors has been presented by Rondoni (74), Dieckmann (75), Euler (76), and Hinsberg (77).

APPLICATION OF PROTEOLYTIC ENZYMES

Hotchkiss (78) has employed enzymatic digestion in an attempt to determine the number of peptide bonds in lactoglobulin.

Damodaran & Ramachadran (79) have reported that on treatment of "paranuclein" (product of the action of pepsin on casein) by means of pancreatin, a phosphopeptone of constant composition is obtained. The authors believe this product to be composed of three glutamic acid residues, three isoleucine residues, four serine residues, and three phosphoric acid residues per unit of ten amino acid residues.

Bernheim (80) has studied the action of papain and pancreatin on several dehydrogenases. Succinoxidase appears to be most sensitive to the action of pancreatin.

Skoog & Thimann (81) have found that chymotrypsin is highly effective in increasing the yield of auxin from plant tissues. The authors conclude that the auxin in *Lemna* is bound to a protein and is liberated from it on enzymatic hydrolysis.

Chow, Greep & van Dyke (82) have studied the action of various proteolytic enzymes on the physiological potency of anterior pituitary extracts. Crystalline carboxypeptidase and papain were found to have no specific effect but probably cause general destruction. Crystalline chymotrypsin and trypsin destroy the luteinizing and thyrotropic hormones; these two enzymes impair the action of the follicle-stimulating hormone. In the experiments of these workers pancreatin was found to destroy only the luteinizing activity. McShan & Meyer (83) have reported that pancreatin destroys the luteinizing, lactogenic, and thyrotropic activity of pituitary extracts. These authors employ enzymatic digestion for the preparation of follicle-stimulating fractions. See also (84).

MacFarlane & Dolby (85) have reported that the rate of peptic hydrolysis of the proteins of normal and immune sera is very similar.

Schultze (86) has reported that some immune sera are more resistant than normal serum to the action of pepsin; however, no simple relationship was found between the extent of hydrolysis by pepsin at pH 4.5 and the antibody titer or cholesterol content. Modern & Ruff (87) have studied the optimal conditions for the purification of anti-diphtheria sera by means of peptic proteolysis.

Weineck (88) has reported that preparations of vaccine virus are not inactivated by the addition of a large variety of proteolytic enzymes. However, Hoagland, Ward, Smadel & Rivers (89), in a careful study of the action of purified proteolytic enzymes on elementary bodies of vaccinia, have found that cyanide-papain rapidly destroys the infectivity and the staining reaction with a rapid liberation of amino nitrogen. On the other hand, crystalline trypsin, crystalline chymotrypsin, crystalline carboxypeptidase, cyanide-ficin, or cyanide-cathepsin cause no significant loss in infectivity or staining properties. Pepsin hydrolyzes the inactivated virus at pH 2 with liberation of amino nitrogen. MacFarlane & Dolby have reported on the enzymatic activity of vaccinial elementary bodies (90).

Shemin, Sproul & Jobling (91) have found that the active agent of the Rous chicken sarcoma can be precipitated by means of papain.

Berger & Asenjo (92) have reported experiments on the digestion of ascaris worms by crystalline papain.

PHYSIOLOGY OF PROTEOLYTIC ENZYMES

Linderstrøm-Lang & Holter (93), by means of micro methods, have studied the distribution of the following proteolytic enzymes in various layers of the mucous membrane of the gastrointestinal tract of several vertebrates: dipeptidase (substrate—alanylglycine), aminopeptidase (substrate—alanylglycylglycine), prolidase (substrate—glycyl-l-proline), and pepsin (substrate—edestin).

Avery & Linderstrøm-Lang (94) have performed a study of the distribution of peptidase activity (substrate—alanylglycine) in the coleoptile of *Avena* and report that in coleoptiles of four millimeter length the enzyme activity per unit weight of tissue, or per cell, is greater at the tip. These authors discuss the possible relationship of this result to the auxin gradient.

Palmer & Levy (95) have studied the properties of dipeptidases (substrate—l-alanylglycine) in chick embryo extracts and have presented data (96) on the accumulation of this type of enzyme during the development of the embryo.

Bliss (97) has reported that treatment of frogs' eggs with pancreatin for a few hours during the early stages of development renders the egg membranes fragile but does not kill the embryo.

Munoz, Braun-Menendez, Fasciolo & Leloir (98) offer evidence in support of their view that renin is a proteolytic enzyme secreted by the ischemic kidney. It is assumed that renin acts on a blood globulin to produce a polypeptide-like substance that has a direct vasoconstrictor action. The authors report that partially purified renin is practically free of cathepsin.

Ramirez de Arellano, Lawton & Dragstedt (99) have confirmed the finding of Rocha e Silva (100) that perfusion of trypsin through guinea pig lung leads to the liberation of histamine. These authors also report that intravenous injection of trypsin into dogs leads to a marked drop in blood pressure.

LITERATURE CITED

1. LINDERSTRØM-LANG, K., *Ann. Rev. Biochem.*, **8**, 37 (1939)
2. BALLS, A. K., *Ann. Rev. Biochem.*, **9**, 43 (1940)
3. HERRIOTT, R. M., DESREUX, V., AND NORTHROP, J. H., *J. Gen. Physiol.*, **23**, 439 (1940)
4. ÅGREN, G., AND HAMMARSTEN, E., *Enzymologia*, **4**, 49 (1937)
5. TISELIUS, A., HENSCHEN, G. E., AND SVENSSON, H., *Biochem. J.*, **32**, 1814 (1938)
6. HERRIOTT, R. M., DESREUX, V., AND NORTHROP, J. H., *J. Gen. Physiol.*, **24**, 213 (1940)
7. HERRIOTT, R. M., DESREUX, V., AND NORTHROP, J. H., *Science*, **92**, 456 (1940)
8. NORRIS, E. R., AND ELAM, D. W., *J. Biol. Chem.*, **134**, 443 (1940)
9. MATTHEWS, J. E., DOW, R. B., AND ANDERSON, A. K., *J. Biol. Chem.*, **135**, 697 (1940)
10. HORWITT, M. K., *Science*, **92**, 89 (1940)
11. GLAZKO, A. J., AND FERGUSON, J. H., *Proc. Soc. Exptl. Biol. Med.*, **45**, 43 (1940)
12. GLAZKO, A. J., AND FERGUSON, J. H., *J. Gen. Physiol.*, **24**, 169 (1940)
13. BUTLER, J. A. V., *J. Gen. Physiol.*, **24**, 189 (1940)
14. ANSON, M. L., *J. Gen. Physiol.*, **20**, 565 (1937)
15. ANSON, M. L., *J. Gen. Physiol.*, **23**, 695 (1940)
16. FREUDENBERG, E., *Enzymologia*, **8**, 385 (1940)
17. WINNICK, T., DAVIS, A. R., AND GREENBERG, D. M., *J. Gen. Physiol.*, **23**, 275 (1940)

18. GREENBERG, D. M., AND WINNICK, T., *J. Biol. Chem.,* **135,** 761, 775 (1940)
19. BALLS, A. K., THOMPSON, R. R., AND JONES, W. W., *Ind. Eng. Chem., Ind. Ed.,* **32,** 1144 (1940)
20. BALLS, A. K., LINEWEAVER, H., AND SCHWIMMER, S., *Ind. Eng. Chem., Ind. Ed.,* **32,** 1277 (1940)
21. DYCKERHOFF, H., AND GIGANTE, D., *Biochem. Z.,* **304,** 334 (1940)
22. HAAS, R., *Biochem. Z.,* **305,** 280 (1940)
23. SCHÖBERL, A., AND RAMBACHER, P., *Biochem. Z.,* **305,** 223 (1940)
24. JOHNSON, M. J., *Rept. Proc. Third Intern. Congr. Microbiol.,* 209 (1939)
25. BERGER, J., AND JOHNSON, M. J., *J. Biol. Chem.,* **133,** 157 (1940)
26. BERGER, J., AND JOHNSON, M. J., *J. Biol. Chem.,* **130,** 641, 655 (1939)
27. MASCHMANN, E., *Naturwissenschaften,* **27,** 819 (1939)
28. MASCHMANN, E., *Biochem. Z.,* **303,** 145 (1939)
29. FRANKENTHAL, L., *Enzymologia,* **8,** 307 (1940)
30. DALE, W. M., *Nature,* **145,** 977 (1940)
31. TISELIUS, A., AND ERIKSSON-QUENSEL, I. B., *Biochem. J.,* **33,** 1752 (1939)
32. FRUTON, J. S., AND BERGMANN, M., *J. Biol. Chem.,* **127,** 627 (1939)
33. TAZAWA, Y., *Enzymologia,* **7,** 321 (1939)
34. BERGMANN, M., AND FRUTON, J. S., *Advances in Enzymology* (In press)
35. ABDERHALDEN, E., *Fermentforschung,* **16,** 182 (1940)
36. FRUTON, J. S., AND BERGMANN, M., *J. Biol. Chem.,* **136,** 559 (1940)
37. UCHINO, T., *J. Biochem. (Japan),* **31,** 323 (1940)
38. NAKAMURA, K., *J. Biochem. (Japan),* **31,** 311 (1940)
39. HOFMANN, J., AND BERGMANN, M., *J. Biol. Chem.,* **134,** 225 (1940)
40. ABDERHALDEN, E., AND ABDERHALDEN, R., *Fermentforschung,* **16,** 48 (1938)
41. UTZINO, S., AND NAKAYAMA, M., *Enzymologia,* **8,** 280 (1940)
42. KAZAMA, T., *Acta Schol. Med. Univ. Imp. Kioto,* **23,** 154 (1939)
43. KAZAMA, T., *Ber.,* **73,** 331 (1940)
44. FRUTON, J. S., AND BERGMANN, M., *J. Biol. Chem.,* **133,** 153 (1940)
45. IRVING, JR., G. W., FRUTON, J. S., AND BERGMANN, M., *J. Biol. Chem.* (In press)
46. MASCHMANN, E., *Biochem. Z.,* **279,** 225 (1935)
47. SCHÖBERL, A., *Angew. Chem.,* **53,** 227 (1940)
48. BERGER, J., AND JOHNSON, M. J., *J. Biol. Chem.,* **133,** 639 (1940)
49. TALCE-NIEDRA, D., *Enzymologia,* **8,** 163 (1940)
50. LINDERSTRØM-LANG, K., AND JOHANSEN, G., *Enzymologia,* **7,** 239 (1939)
51. MAVER, M. E., AND VOEGTLIN, C., *Enzymologia,* **6,** 219 (1939)
52. STRAIN, H. H., *Enzymologia,* **7,** 133 (1939)
53. ÅGREN, G., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **23,** 173 (1940)
54. ÅGREN, G., AND HAMMARSTEN, E., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **22,** 25 (1938)
55. STRAIN, H. H., AND LINDERSTRØM-LANG, K., *Enzymologia,* **7,** 241 (1939)
56. COLLIER, H. B., *Can. J. Research, B,* **18,** 255 (1940)
57. COLLIER, H. B., *Can. J. Research, B,* **18,** 272 (1940)
58. COLLIER, H. B., *Can. J. Research, B,* **18,** 305 (1940)
59. FRUTON, J. S., IRVING, JR., G. W., AND BERGMANN, M., *J. Biol. Chem.,* **133,** 703 (1940)

60. CHEN, T. F., *Chinese J. Physiol.*, **15**, 159 (1940)

61. WALDSCHMIDT-LEITZ, E., AND MAYER, K., *Z. physiol. Chem.*, **262**, IV (1939)

62. BAYERLE, H., AND PODLOUCKY, F. H., *Biochem. Z.*, **304**, 259 (1940)

63. BAYERLE, H., AND PODLOUCKY, F. H., *Z. physiol. Chem.*, **264**, 189 (1940)

64. WALDSCHMIDT-LEITZ, E., MAYER, K., AND HATSCHEK, R., *Z. physiol. Chem.*, **263**, I (1940)

65. WALDSCHMIDT-LEITZ, E., AND HATSCHEK, R., *Z. physiol. Chem.*, **264**, 196 (1940)

66. EULER, H. VON, AND SKARZYNSKI, B., *Arkiv Kemi Mineral. Geol., B*, **14**, No. 11 (1940)

67. EULER, H. VON, AND SKARZYNSKI, B., *Z. physiol. Chem.*, **265**, 133 (1940)

68. ABDERHALDEN, E., AND ABDERHALDEN, R., *Z. physiol. Chem.*, **265**, 253 (1940)

69. HERKEN, H., AND ERXLEBEN, H., *Z. physiol. Chem.*, **264**, 251 (1940)

70. FRUTON, J. S., IRVING, JR., G. W., AND BERGMANN, M., *J. Biol. Chem.*, **132**, 465 (1940)

71. ABDERHALDEN, E., AND CAESAR, G., *Fermentforschung*, **16**, 299 (1940)

72. BAYERLE, H., *Biochem. Z.*, **303**, 251 (1939)

73. BAYERLE, H., *Biochem. Z.*, **305**, 227 (1940)

74. RONDONI, P., *Ergeb. Hyg. Bakt. Immunitätsforsch. Exptl. Therap.*, **23**, 1 (1940)

75. DIECKMANN, H., *Z. Krebsforsch.*, **50**, 41 (1940)

76. EULER, H. VON, *Angew. Chem.*, **53**, 352 (1940)

77. HINSBERG, K., *Angew. Chem.*, **53**, 355 (1940)

78. HOTCHKISS, R. D., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **23**, 125 (1939)

79. DAMODARAN, M., AND RAMACHADRAN, B. V., *Nature*, **145**, 857 (1940)

80. BERNHEIM, F., *J. Biol. Chem.*, **133**, 141 (1940)

81. SKOOG, F., AND THIMANN, K. V., *Science*, **92**, 64 (1940)

82. CHOW, B. F., GREEP, R. O., AND VAN DYKE, H. B., *J. Endocrinol.*, **1**, 440 (1939)

83. McSHAN, W. H., AND MEYER, R. K., *J. Biol. Chem.*, **135**, 473 (1940)

84. LI, R. C., *Proc. Soc. Exptl. Biol. Med.*, **43**, 598 (1940)

85. MACFARLANE, M. G., AND DOLBY, D. E., *Chemistry & Industry*, **59**, 730 (1940)

86. SCHULTZE, H. E., *Biochem. Z.*, **305**, 196 (1940)

87. MODERN, F., AND RUFF, G. R., *Biochem. Z.*, **305**, 405 (1940)

88. WEINECK, E., *Naturwissenschaften*, **28**, 171 (1940)

89. HOAGLAND, C. L., WARD, S. M., SMADEL, J. E., AND RIVERS, T. M., *J. Exptl. Med.*, **72**, 685 (1940)

90. MACFARLANE, M. G., AND DOLBY, D. E., *Brit. J. Exptl. Path.*, **21**, 219 (1940)

91. SHEMIN, D., SPROUL, E. E., AND JOBLING, J. W., *J. Exptl. Med.*, **72**, 697 (1940)

92. BERGER, J., AND ASENJO, C. F., *Science*, **91**, 387 (1940)

93. LINDERSTRØM-LANG, K., AND HOLTER, H., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **23**, 135 (1940)

94. AVERY, JR., G. S., AND LINDERSTRØM-LANG, K., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **23,** 219 (1940)
95. PALMER, A. H., AND LEVY, M., *J. Biol. Chem.,* **136,** 407 (1940)
96. LEVY, M., AND PALMER, A. H., *J. Biol. Chem.,* **136,** 415 (1940)
97. BLISS, A. F., *Proc. Soc. Exptl. Biol. Med.,* **43,** 769 (1940)
98. MUNOZ, J. M., BRAUN-MENENDEZ, E., FASCIOLO, J. C., AND LELOIR, L. F., *Am. J. Med. Sci.,* **200,** 608 (1940)
99. RAMIREZ DE ARELLANO, M., LAWTON, A. H., AND DRAGSTEDT, C. A., *Proc. Soc. Exptl. Biol. Med.,* **43,** 360 (1940)
100. ROCHA E SILVA, M., *Arq. Inst. biol.,* **10,** 93 (1939)

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NONPROTEOLYTIC ENZYMES

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Esterases

Liver esterase.—Kraut & Pantschenko-Jurewitz (1) have reported that ascorbic acid is a part of the liver esterase complex. Their findings have been confirmed by Goldstein & Bondareva (2) who claim that liver esterase, when oxidized with ascorbic acid oxidase, showed considerable decrease in esterase activity and that the ascorbic acid present in the rat liver suspension employed as the enzyme source was almost completely oxidized. Kertesz (3), however, purified pig liver esterase by adsorption on lead phosphate and fractional precipitation with ammonium sulfate. This preparation could not be inactivated by carbon, kieselguhr, fuller's earth, or acid clay, or by dialysis. The purified esterase of Kertesz did not reduce dichlorophenolindophenol. Thus he concluded that ascorbic acid is not a coenzyme of the esterase.

Muscle esterase.—Matlack & Tucker (4) investigated meat muscle esterase. Most of the enzyme was found to be bound to insoluble muscle proteins. By ammonium sulfate precipitation of alkaline extracts a soluble esterase was obtained which resembled liver esterase. It hydrolyzed only glycerides and other esters of the lower fatty acids. Lecithinase, cholinesterase, and phosphatase were not present in the esterase preparation. The enzyme had an optimum activity at pH 6.0 in the presence of phosphate. Bile inhibited the esterase.

Red cell lipase.—Scoz (5) showed that oxalic acid and citric acid increase the activity of red cell lipase 400 per cent. The enzyme has an optimum activity at pH 8.0.

According to Schramm & Wolff (6) acetone preparations of pancreas and glycerol extracts thereof readily esterify cholesterol, dehydroandrosterone, dihydrocholesterol, sitosterol, stigmasterol, and ergosterol.

Cholinesterase.—Massart & Dulfait (7) reported that cholinesterase was greatly inhibited by sodium oxalate, citrate, fluoride, arsenite, and pyrophosphate in 0.02 M concentrations. Sodium thiocyanate and sodium cyanide did not inhibit the enzyme. When horse serum was dialyzed against dilute sodium bicarbonate, 40 per cent

of the cholinesterase activity was lost. It was restored again, however, on the addition of calcium, magnesium, or manganese ions. Alles & Hawes (8) found that the cholinesterase of human blood serum and that of blood cells are dissimilar in respect to substrate specificity, activity-pH relationship, and effect of salt content of the medium. Studies of either enzyme alone are inadequate, according to these authors, in evaluating the cholinesterase activity of whole blood. Similar findings are reported by Schramm & Wolff (9).

Glick & Antopol (10) showed that cholinesterase was less inhibited by thiamin chloride acetic ester than by free thiamin, and that cocarboxylase was the least inhibitory. Thiamin chloride acetic ester was slowly hydrolyzed by horse serum. Süllmann & Birkhäuser (11) found that thiamin chloride acetic ester was not hydrolyzed by horse serum rich in cholinesterase.

Tropine esterase.—Glick (12) studied the hydrolysis of atropine by rabbit serum tropine esterase. The reaction was found to be of zero order. The optimum pH of the enzyme is 8.1 to 8.4 at 38° C. The value of the Michaelis constant, 6×10^{-5}, indicates an unusually great affinity between substrate and enzyme. The temperature coefficients were 2.0 for the range of 25° to 35° and 1.9 for 28° to 38°C. Glick suggests that tropine esterase and cholinesterase are probably separate enzymes.

THE ENZYMATIC SYNTHESIS AND HYDROLYSIS OF STARCH AND GLYCOGEN

Dahl (13) reports that phlorhizin and phloretin inhibit the phosphorylation of starch and of glycogen by muscle extracts. Other glucosides, such as arbutin and salicin, however, do not inhibit phosphorylation. The aglucone part of the glucosides is responsible for the inhibition. Pyrocatechol also inhibits the phosphorylation of glycogen and starch. Glucose exhibits inhibition in high concentrations only. Inhibition by phlorhizin or glucose affects the formation of the Cori ester. The phosphoglucosemutase system is not affected by the inhibitors.

Euler & Bauer (14) showed that alumina-Cγ adsorbs starch phosphorylase at pH 5.0 and 8.5 from muscle extracts. The enzyme may be eluted from the gel with phosphate at pH 7.0. Adenylic acid and manganese and magnesium ions were necessary for phosphorylation.

Willstätter & Rohdewald (15) found that glycogen synthesis from

glucose is an intermediary step in the formation of lactic acid *in vitro*. Glycogen formation from glucose by minced muscle required hexokinase. Without hexokinase a polysaccharide different from glycogen was formed.

Colowick, Welch & Cori (16) investigated the phosphorylation of glucose (an intermediary reaction in glycogen formation) by cell-free liver and kidney extracts. Dialyzed or aged extracts required at least two coenzymes, adenylic acid and cozymase (diphosphopyridine nucleotide), magnesium ions, and an oxidizable substrate such as a dicarboxylic acid. Kalckar's findings were confirmed, showing that for the esterification of glucose with inorganic phosphate in cell-free kidney extracts aerobic conditions are necessary.

Cori & Cori (17), employing highly purified muscle, brain, heart, and liver enzymes, studied the kinetics and specific conditions requisite for glycogen synthesis from glucose-1-phosphate. For either direction of the reaction—glycogen formation or hydrolysis—the coenzyme, adenylic acid, and glycogen are essential. It was found that inosinic acid cannot replace adenylic acid in the above system. Reducing agents such as glutathione increase the activity of the purified phosphorylase; for full activity, it is necessary that the reducing agents act on the phosphorylase. Inhibition by traces of copper is counteracted by the reducing agents. The position of the equilibrium is governed by the pH only. The reaction rate is unimolecular at the pH optimum (6.3 to 6.5). α-Glucose, phlorhizin, and phloretin inhibit phosphorylase activity in both directions. β-Glucose, fructose, mannose, glucose-6-phosphate, and maltose inhibit the activity only slightly. Süllmann (18), contrary to the findings of Cori & Cori (17), claims that inosinic acid can replace adenylic acid in the phosphorylation of glycogen and glucose. Both carbohydrates produce lactic acid in the presence of inosinic acid.

Hanes (19) prepared a purified phosphorylase from potato tuber juice, which reversibly converted glucose-1-phosphate to starch and free phosphate at pH 5.4. He isolated 20 gm. of the synthetic starch and found it to be identical with natural starch in all of the properties examined.

Somogyi (20) confirmed the earlier findings of other investigators that, in addition to reducing dextrins and maltose, glucose is a normal product of amylolytic activity.

McClure (21) showed that sodium fluoride, potassium fluoride, ammonium fluoride, sodium fluosilicate have no effect on salivary

amylase. Ninomiya (22) purified salivary amylase. The enzyme is rapidly digested by pepsin at pH 4.4, but more slowly by trypsin at pH 8.0. Potassium cyanide and potassium thiocyanate, even in a concentration of 0.0005 M, inhibit the activity of salivary amylase. Ballou & Luck (23) investigated the saccharogenic action of taka-diastase as influenced by various buffers. The relative activity of the amylase at the pH optimum and on the alkaline side of the optimum was not affected by changing the buffer anion; marked differences occurred on the acid side, however. Janke & Holota (24) studied extensively the kinetics of amylolytic action.

OTHER CARBOHYDRASES

Hestrin (25) found that crude taka-diastase preparations hydrolyzed maltose, maltosazone, maltobionic acid, sucrose, raffinose, lactose, and salicin. Methyl α-glucoside, however, was only slightly hydrolyzed even by very high concentrations of enzyme. Taka-maltase is much more heat stable than the other taka-carbohydrases. Taka-maltase is acid resistant, whereas sucrase is not. Other data are presented to support the theory of Leibowitz. The experimental findings of Hestrin are in contradiction to Weidenhagen's theory of the specificity of carbohydrases.

According to Bauer (26) yeast invertase is inhibited by methylene blue, acridine yellow, and other dyes. Aniline, naphthylamine derivatives, allylthiourea, and barbiturates also inhibit the enzyme.

Freidenberg & Ploetz (27) separated the cellulase and lichenase of *Aspergillus oryzae* using a mixture of alcohol and ether; the lichenase was precipitated first, the cellulase next. The remaining liquid contained cellobiase. Similar experiments were conducted with the digestive juice of *Helix pomatia.*

Malaguzzi-Valeri (28) showed that by dialyzing an emulsin preparation at its isoelectric point (pH 6.1) or lower, the enzyme may be separated into an inactive apoenzyme and an inactive coenzyme. The two may be reunited to form an active enzyme. The coenzyme is neither a polypeptide nor an amino acid. It does not contain amino, sulfhydryl, or sulfo groups. The holoenzyme in the anion form is stable. It splits spontaneously into the coenzyme and the apoenzyme when it is present as a cation.

Robertson, Ropes & Bauer (29) investigated the enzyme mucinase which they obtained from broth cultures of *Clostridium perfringens.*

A 900-fold concentration was attained by adsorption of the enzyme on calcium phosphate from a 50 per cent acetone solution. Mucinase decreases the viscosity of a synovial fluid mucin or of the prosthetic polysaccharide; amino sugars and other reducing substances are thereby liberated. The enzyme is active between pH 3.9 and 8.5. Its average temperature coefficient (K_{10}) is 1.75 and its inactivation takes place at 60° C. Removal of salts from the enzyme solution causes reversible inactivation. Cyanide, arsenite, and iodine, however, inactivate the enzyme irreversibly.

Claud (30) reported that leech extracts contain a powerful mucinase, which has a strong effect on the viscosity of chicken tumor extracts and of solutions of rabbit skin mucoprotein. He suggests that mucinase and the "spreading" factor may be identical.

Fishman (31) applied the enzyme β-glucuronidase to the assay of estriol glucuronide in urine. Estriol glucuronide is a normal constituent of the animal body.

COPPER-PROTEID OXIDASES

Ascorbic acid oxidase.—Mituda (32) prepared ascorbic acid oxidase from pumpkin by ammonium sulfate precipitation. This preparation oxidized *l*-ascorbic acid but not *l*-cysteine. Tadokoro & Takasugi (33) obtained ascorbic acid oxidase in crystalline form on the addition of ammonium sulfate to aqueous pumpkin extract. The enzyme was found to be an albumin and was very active. Stotz (34) made the remarkable observation that glycine at pH 6.0 completely inhibits the action of nonenzymic copper, but not that of the copper-protein enzyme. This enzyme preparation was obtained from cucumbers by barium acetate clarification, ammonium sulfate precipitation, fractional heat denaturation, dialysis, and fractionation with ammonium sulfate. The final product was highly active and contained 0.25 per cent copper.

Lovett-Janison & Nelson (35) have also isolated a highly active ascorbic acid oxidase. Their enzyme was obtained from summer squash and is a copper protein having only 0.15 per cent copper. Solutions of the enzyme are green in high concentration and blue in low concentration. Ramasarma, Datta & Doctor (36) found copper to be an active constituent of ascorbic acid oxidase. Not all the copper present in vegetable juices is enzymic, however.

Tyrosinase.—Gregg & Nelson (37), using an enzyme preparation obtained from the common mushroom *Psalliota campestris,* confirmed earlier findings published from their laboratory. Tyrosinase cannot oxidize *p*-cresol without the simultaneous oxidation of an *o*-dihydric phenol. In the presence of *p*-cresol tyrosinase does not oxidize catechol any faster than is necessary for priming the enzyme toward *p*-cresol. Tyrosinase preparations with high cresolase activity showed that both catechol oxidation and *p*-cresol oxidation are proportional to the same copper content. According to these authors, this is evidence indicating that catecholase and cresolase activity belong to the same "tyrosinase" enzyme complex. The same investigators (38) showed that the highly purified tyrosinase which they obtained from *Psalliota campestris* acted on both monohydric phenols and polyphenols as well. Tyrosinase activity is proportional to the copper content of the enzyme. The activity of the enzyme, however, varies with the substrate.

Laccase.—Gregg & Miller (39) studied the enzyme of the wild mushroom *Russula foetens.* It oxidized aerobically hydroquinone and *p*-phenylenediamine. When aqueous extracts of dried mushroom powder were treated with acetone, the laccase precipitated out, whereas the tyrosinase was destroyed. Laccase catalyzes the aerobic oxidation of a great variety of substrates, and the authors point out that this fact should not cause confusion with other oxidases. Laccase is inhibited by cyanides. Keilin & Mann (40) found that the laccases of laquer trees from Indo-China, Japan, and Burma are all copper-protein complexes containing a blue pigment; the latter is now under investigation.

Other Oxidases

Unsaturated fat oxidase.—In 1928 Bohn & Haas found that legume seeds, such as soybean and navy bean, contain an enzyme which oxidizes carotene and unsaturated fats. They obtained patents for bleaching wheat flour by this method. The reaction is a very interesting one. The intense reddish brown color of carotene disappears within a few minutes when shaken with a suspension of ground soybeans. In 1935 Hauge (41) showed that alfalfa contains a vitamin-A-destroying enzyme, and Wilbur and associates (42) demonstrated that soybeans in the ration of dairy cows had a destructive effect on the vitamin-A value of butter. Frey and collaborators (43) found that the vitamin-A potency of carotene was completely destroyed when exposed

to the soybean enzyme and that 99 per cent of the vitamin A present in cod liver oil was also inactivated by the enzyme.

Kirsanova (44) reported finding a carotene oxidase in radish juice and in potato juice.

Sumner & Dounce (45) studied extensively the carotene oxidase of soybeans and reported that besides carotene, egg-yolk xanthophyll is also oxidized by the oxidase (the pure pigments were not employed in any of these studies).

More recently Tauber (46) showed that carotene oxidase, as described by these various authors, does not exist and that the oxidation of carotene is caused indirectly by an unsaturated fat oxidase. The unsaturated fats are oxidized (perhaps to peroxides) by the unsaturated fat oxidase and the products of oxidation in turn oxidize the carotenoids. Simultaneously and independently of this report, Sumner & Sumner (47) came to similar conclusions.

Fatty acid dehydrogenase.—According to Lang & Adickes (48), stearic acid is converted to palmitic acid by a dehydrogenase; they claim that the earlier conception of the oxidation of the fatty acid is incorrect.

Phosphatases

Hove and co-workers (49) found crude intestinal phosphatase to increase in activity 40 to 100 per cent *in vitro* on the addition of zinc ions. Crude kidney and bone phosphatases are inhibited under the same conditions. After dialysis, however, all three enzymes showed marked inhibition by zinc. A dialyzate from intestinal phosphatase restored the ability of zinc to activate the dialyzed enzyme. The zinc coactivator, according to the authors, is a product of mucosal tissue autolysis. Several α-amino acids have been tried and they all have the same coactivating property as the dialyzate. β-Amino, keto, hydroxy, or aliphatic acids or organic amines have little or no activity. It is suggested that α-amino acids may play a role as phosphate transporters in dephosphorylation.

Weil & Russell (50) demonstrated that plasma phosphatase activity is greatly influenced by the diet; fasting decreases plasma phosphatase activity. Ingestion of carbohydrates and proteins did not increase the low plasma phosphatase activity brought about by fasting. Only the ingestion of the fraction of the rat diet which was soluble

in alcohol-ether caused an increase in the phosphatase concentration. Cephalin produced a marked increase, whereas lecithin was without effect. Eight per cent lard in the diet produced the highest phosphatase activity.

Travia & Veronese (51) found that the phosphoric acid radical may be completely liberated from casein by phosphatase in six hours.

Thiamin Pyrophosphate Proteids and Cocarboxylase

In 1913, Neuberg & Kerb discovered the enzyme carboxylase in yeast and in other plants. Yeast carboxylase is an important enzyme. It converts pyruvic acid into acetaldehyde and, from acetaldehyde, alcohol is formed. More recently a specific pyruvic acid oxidase system, acting in a different manner from yeast carboxylase, has been recognized in bacteria and in mammalian tissues. Owing to their important functions and their interaction with cocarboxylase (thiamin pyrophosphoric acid ester) these enzyme systems have been extensively investigated and many important papers have appeared recently.

Synthetic cocarboxylase.—In 1932 Auhagen was the first to show that yeast "carboxylase" can be separated into two components, one a protein and the other a thermostable principle. Simola in the same year noted a connection between thiamin and carboxylase. Kinnersley & Peters had shown as early as 1928 that there was a form of thiamin, precipitable by lead acetate, in yeast, and in 1937 Lohmann & Schuster (52) isolated cocarboxylase from yeast in pure crystalline form. They showed that it was the pyrophosphoric acid ester of thiamin. One year later Tauber (53), using a new method for the synthesis of primary esters of phosphoric acid, obtained cocarboxylase synthetically by heating synthetic thiamin and anhydrous sodium pyrophosphate in a medium of dehydrated orthophosphoric acid (containing mainly pyrophosphoric acid). The pyrophosphoric acid ester was isolated in pure and crystalline state by Weijlard & Tauber (54). Hydrolysis and cleavage products showed that the synthetic coenzyme was in every respect identical with the natural substance. Thus, Lohmann & Schuster's important findings were fully confirmed by synthesis and analysis of the synthetic product.

Weil-Malherbe (55) has now published a new synthesis of the coenzyme. He states, however, that the method of Weijlard & Tauber is preferable because of its greater simplicity and recommends certain

modifications in the purification procedure. In the method of Weil-Malherbe the alcoholic hydroxyl group of thiamin was replaced with bromine by heating with alcoholic hydrogen bromide. The bromo-thiamin was then made to react with silver pyrophosphate in pyro-phosphoric acid. The mono- and diphosphates were separated by means of phosphotungstate precipitation, silver precipitation, and re-crystallization from aqueous acetone and alcohol.

Weil-Malherbe compared the cocarboxylase synthesized by the bromothiamin method with that prepared by him by the Weijlard-Tauber procedure, and with a sample of Merck's synthetic cocar-boxylase. All three products were found to be pure as shown by elementary analysis. The catalytic activities of the samples, though equal among themselves, were 40 per cent below that of natural co-carboxylase. The activity findings are no doubt correct. Earlier, Ochoa (56), reporting from the Oxford laboratory (in which the activity tests, just mentioned, were carried out), had made the very important observation, however, that unphosphorylated thiamin has a strong activating effect on cocarboxylase. Obviously, the question arises whether the natural cocarboxylase was completely free of traces of unphosphorylated thiamin.

The soluble carboxylase recently described by Weil-Malherbe (57) is not accelerated by thiamin in the presence of cocarboxylase even in concentrations of cocarboxylase far below saturation and should be most useful in testing cocarboxylase activity. There is no statement in any of these papers which would indicate that the new carboxylase was employed in these tests.

Cocarboxylase in plants and mammalian tissues.—Cocarboxylase is widely distributed in animal (58) and plant tissues. Tauber (59) found cocarboxylase to be present in a variety of plants such as onions, pecans, green peppers, spinach, summer squash, turnip greens, oranges (juice, peel, and seeds), and in the mycelium of *Aspergillus niger*. In this paper a typographical error occurred. The thiamin content should read "per 100 grams of plants." The cocarboxylase content as given per gram of plants is correct, however. Considering the possibility of cocarboxylase activation, owing to the presence of free thiamin in these plants, the cocarboxylase values, as recorded by Tauber, are apparently too high. Hennessy & Cerecedo (60) have determined the content of free and phosphorylated thiamin in several foods, urine, and blood, by a modified thiochrome method combined with hydrolysis of the cocarboxylase by phosphatase.

The indispensability of cocarboxylase for pyruvic acid oxidation in various cells is now definitely established (61, 62, 63) and it is well known that pyruvic acid is an important metabolic link. A series of products, varying with the organism and the tissues, has been shown to result from pyruvic acid oxidation. For an excellent discussion and an extensive bibliography concerning this subject the recent paper by Sober, Lipton & Elvehjem (64) should be consulted.

The carboxylase enzyme system.—Lohmann & Kossel (65) studied the effect of metals on the carboxylase enzyme system. Zinc in low concentrations inhibited cocarboxylase-free carboxylase; in high concentrations it activated the enzyme, whereas in still higher concentrations it inhibited reversibly. The effect of zinc was especially pronounced when the yeast was washed with acid phosphate but, in the presence of manganese ions, the effect was compensated for and the zinc acted additively. While magnesium, manganese, and iron activated, copper, mercury, gold, aluminum, chromium, beryllium, lead, strontium, and barium inhibited the carboxylase enzyme system. Zinc inhibited the formation of lactic acid by aqueous muscle extracts. Lohmann & Kossel believe that zinc has a definite role in carbohydrate metabolism.

The enzyme carboxylase has not yet been obtained in the pure state owing to its instability when freed of cocarboxylase and of activators such as manganese and magnesium. According to Melnick & Stern (66), frozen ammonium sulfate extracts of yeast carboxylase are more stable than water and phosphate extracts, provided they are kept in a frozen state at low temperatures. Even ammonium sulfate extracts, however, were found to lose their activity when allowed to stand at room temperature. The reviewer (67) found that cocarboxylase protects carboxylase for some time from rapid inactivation. Carboxylase is most active at pH 6.1, and has an isoelectric point of 5.1 (66). Melnick & Stern found that phosphopyruvic acid is not an intermediary in the decarboxylation of pyruvic acid by carboxylase. Ultracentrifuge experiments of Melnick & Stern indicate that one molecule of carboxylase catalyzes the reaction of many cocarboxylase and substrate molecules by rapidly exchanging its prosthetic group during catalysis. Green and co-workers (68) showed that the molecular ratio of carboxylase enzyme protein to cocarboxylase, is 1:1, and of cocarboxylase to magnesium, 1:5. They have obtained the enzyme-coenzyme-magnesium complex in a highly purified stable state from top fermenting brewers' yeast by salt fractionation. The carboxylase

enzyme complex contained 0.46 per cent cocarboxylase and 0.13 per cent magnesium. One mg. of the enzyme complex catalyzed the formation of 12.1 cc. of carbon dioxide per hour at 30° C.

Greenberg & Rinehardt (69) found that cysteine, reduced glutathione, sodium bisulfite, and phenylhydrazine increased the activity of the carboxylase enzyme system. The activation is apparently based on the formation of more reactive addition (enol) compounds with pyruvic acid, as observed by Tauber (67) in the case of sodium cyanide. These compounds also combine with the carboxylase-inhibitory end product, acetaldehyde.

Buchman and associates (70) found that while several substances with a structure similar to that of cocarboxylase, but without cocarboxylase activity, were able to inhibit considerably the activity of the carboxylase enzyme system, the thiazole pyrophosphate portion of the cocarboxylase molecule was the most active inhibitor. The inhibition is said to be caused by competition between cocarboxylase and the thiazole pyrophosphate for the specific carboxylase protein, with which both are able to combine.

Westenbrink & van Dorp (71) reported that the activation of cocarboxylase by thiamin, observed by Ochoa (56), is caused by the inhibitory effect of thiamin on the cocarboxylase-hydrolyzing phosphatase. The latter is active in the alkali-washed yeast which is employed as the source of carboxylase. Lipton & Elvehjem (72), however, showed that the activation of cocarboxylase by thiamin depends upon the kind of yeast used in the test. Brewers' yeast exhibited the effect only very slightly, whereas with bakers' yeast the activating effect was very strong. The observation of Lipmann, and of Westenbrink & van Dorp, that excess thiamin accelerates cocarboxylase action only when added to carboxylase before or simultaneously with cocarboxylase, was confirmed.

Lipton & Elvehjem (72) furnished a solution to the problem of the Ochoa effect. They claim that there is a heat-labile substance present in bakers' yeast which adsorbs cocarboxylase, preventing the production of an active enzyme. The heat-labile substance, if first saturated with thiamin, permits the combination of cocarboxylase with the active carboxylase, a very important observation indeed. The chemical nature and general properties of the heat-labile substance should be further investigated, however.

Enzymic pyrophosphorylation of thiamin.—Weil-Malherbe (57) demonstrated that adenylpyrophosphate greatly accelerates the pyro-

phosphorylation of thiamin. Thiamin monophosphate is not an inter-
mediary. Phosphopyruvic acid cannot act as a phosphate donator in
the presence of small amounts of adenylic acid or adenylpyrophosphate.
According to Weil-Malherbe

The synthesis of cocarboxylase by yeast stops when the carboxylase protein is
partly or fully saturated. No preparative use can therefore be made of the
reactions described.

Larger amounts of thiamin, according to this author, inhibit enzymic
cocarboxylase synthesis. Similar observations were made by the re-
viewer (73). Lipton & Elvehjem (74) found that phosphoglyceric
acid accelerated the enzymic phosphorylation of thiamin in the pres-
ence of adenylic acid and cozymase. Glucose and iodoacetate inhibited
the reaction.

Liebknecht (75) reported that brewers' yeast contains ten to
twenty times more cocarboxylase than bakers' yeast. He found the
cocarboxylase content of bakers' yeast to be very variable, some
bakers' yeast containing no cocarboxylase at all. Fermenting bakers'
yeast, however, showed an energetic synthesis of cocarboxylase soon
after glucose addition.

Attempts have been made to elucidate the mechanism of cocar-
boxylase action in the systems of pyrophosphate proteids (76) and
theoretical considerations have been proposed (77).

Carbonic Acid Anhydrase

Goor (78) investigated the carbonic acid anhydrase content of
tissues of a great number of various animals and described a practical
method for the estimation of the enzyme.

Keilin & Mann (79) published methods for the purification and
isolation of carbonic acid anhydrase from red blood cells and the gas-
tric mucosa of mammals. The purest preparation, believed to be the
enzyme itself, is a colorless protein containing 14.95 per cent nitrogen
and 0.33 per cent zinc. Small amounts of potassium cyanide, hydro-
gen sulfide, and sodium azide reversibly inhibit the enzyme. There is
considerable evidence presented to indicate that the carbonic acid an-
hydrase of Keilin & Mann is very pure and is a zinc proteid. Hove,
Elvehjem & Hart (80) confirmed the findings of Keilin & Mann. Their
enzyme preparation contained 0.3 per cent of zinc. All of the zinc
present in the red blood cells was found to be bound to this enzyme.

Hove and associates showed that the zinc reagent, dithizone, did not significantly inhibit the enzyme. Potassium thiocyanate, however, had a marked inhibitory action and large amounts of zinc also inhibited the carbonic anhydrase.

According to Mann & Keilin (81), sulfanilamide very powerfully inhibits carbonic anhydrase even in concentrations as low as 2×10^{-6} mol. The inhibition is caused by the sulfonamide group. Catalase, peroxidase, cytochrome oxidase, urease, and carboxylase were not affected. The inhibitory action of sulfanilamide is said to be specific for carbonic acid anhydrase.

Kiese & Hastings (82) purified beef erythrocyte carbonic anhydrase. Inhibition was found to be brought about by oxidizing agents such as potassium permanganate, iodine, periodate, perchlorate, chlorate, persulfate, and bromates. The activity could be restored again on the addition of reduced glutathione, cysteine, and hydroquinone. Sulfide and cyanide strongly inhibited the enzyme.

Davenport (83) found the parietal cells of the gastric mucosa a much better source of carbonic acid anhydrase than red blood cells. The chief cells, however, were found to be free of the enzyme.

OTHER ENZYMES

Arginase.—Kitagawa (84) showed that arginase was inhibited by canavanine, arginine, and a number of amino acids; *dl-α*-aminovaleric acid inhibited the enzyme very strongly, being much more inhibitory than the other substances investigated. Fatty acids, acyl derivatives, esters of α-amino acids, and urea had no inhibitory effect. Combination of the amino acids with arginase was proved by estimation of the dissociation constant.

Richards & Hellerman (85) described the preparation of a purified liver arginase of high activity. The enzyme was found to contain manganese or possibly iron as an essential metallic constituent. Purification resulted in a lowering of the metal content. Of the metals originally present only manganese and, to a lesser extent, iron, restored enzyme activity.

Cysteine desulfurase.—Fromageot & Moubasher (86) obtained an enzyme preparation from *B. coli* that produced hydrogen sulfide from cysteine, in the presence of formic, lactic, fumaric, aspartic, or glutamic acids, or glucose. The reaction was inhibited by toluene or fluorides in the presence of formic acid, and by cyanide in the presence of glu-

cose. Citric acid was also inhibitory. The optimum pH of the enzyme was found to be at 6.4 to 6.6.

Heparinase.—Jaques (87) prepared heparinase from rabbit liver by extraction with glycerol and precipitation by half saturation with ammonium sulfate. This enzyme has an optimum pH at 5.3 to 6.8. The mechanism of destruction of heparin by heparinase is not yet known.

Crystalline ribonuclease.—Kunitz (88) obtained ribonuclease in crystalline form from fresh beef pancreas by fractional ammonium sulfate precipitation. The enzyme is an albumin having a molecular weight of 15,000 and an isoelectric point at pH 8.0. Denaturation of the enzyme protein by heat, alkali, or peptic digestion caused a corresponding loss in activity. The ribonuclease is very resistant to heat over a wide pH range and has a maximum heat stability at pH 2.0 to 4.5. It decomposes yeast nucleic acid into readily diffusable products. Free acid groups are formed but only small amounts of phosphoric acid are liberated.

Chlorophyllase.—Weast & Mackinney (89) found that chlorophyllase splits chlorophyll in 80 per cent ethyl alcohol, in 40 to 70 per cent acetone at 25°, or in water at 75°. The results differ, however, with the plant used. According to these authors, the sunflower plant contains an enzyme system capable of hydrolyzing 80 per cent of the chlorophyll in the leaf in thirty minutes in acetone. Certain other plants, on the other hand, showed no chlorophyllase activity.

Crystalline catalase.—Sumner, Dounce & Frampton (90) published an improved method for the preparation of crystalline catalase from horse liver. They found that the catalase activity of various preparations follows inversely the ratio of "blue iron" to hemin iron. The blue substance of crystalline catalase represents changed hematin prosthetic groups which lower the catalase activity. This confirms the work of Lemberg. It is concluded that catalase activity depends upon the number of hematin groups in the enzyme molecule. The total iron content was found to be about 0.09 per cent. This included the hemin iron and "blue iron."

Histidine decarboxylase and histaminase.—Both of these enzymes are present in liver tissue and in other organs. While histidine decarboxylase converts nontoxic amino acids into toxic ones, histaminase and tyramine oxidase in turn change the toxic amines into nontoxic substances.

According to Werle (91) histidine decarboxylase, as well as

histaminase, contains a carbonyl group as indicated by the reversible inhibition produced by various ketone reagents. Histaminase, however, is much more sensitive to these reagents. Girard's reagent inhibited histaminase completely, but histidine decarboxylase only slightly. Histidine decarboxylase, on the other hand, is totally inhibited by germanin, trypan blue, and bile, whereas histaminase is not affected.

Torisu (92) studied the mechanism of histaminase action. He found that histaminase oxidizes and deaminates histamine. Although ammonia is split off, the imidazole nucleus is not affected. Under certain conditions a condensation of the imidazole ring may take place. Urea is not formed in the course of the reaction. Werle & Boden (93) could not find histaminase in yeast, potatoes, or cucumbers.

Transaminase.—Cohen (94, 95) investigated the activity of transaminase with different α-amino acids and α-keto acids.

LITERATURE CITED

1. KRAUT, H., AND PANTSCHENKO-JUREWITZ, W., *Biochem. Z.,* **275,** 114 (1934)
2. GOLDSTEIN, B. I., AND BONDAREVA, S. P., *Ukraïn. Biokhem. Zhur.,* **12,** 91 (1938)
3. KERTESZ, Z. I., *Arkiv Kemi Mineral. Geol., B,* **12,** No. 57 (1938)
4. MATLACK, M. B., AND TUCKER, I. W., *J. Biol. Chem.,* **132,** 663 (1940)
5. SCOZ, G., *Enzymologia,* **7,** 82 (1939)
6. SCHRAMM, G., AND WOLFF, A., *Z. physiol. Chem.,* **263,** 73 (1940)
7. MASSART, L., AND DULFAIT, R., *Bull. soc. chim. biol.,* **21,** 1039 (1939)
8. ALLES, G. A., AND HAWES, R. C., *J. Biol. Chem.,* **133,** 375 (1940)
9. SCHRAMM, G., AND WOLFF, A., *Z. physiol. Chem.,* **263,** 61 (1940)
10. GLICK, D., AND ANTOPOL, W., *Proc. Soc. Exptl. Biol. Med.,* **42,** 396 (1939)
11. SÜLLMANN, H., AND BIRKHÄUSER, H., *Schweiz. med. Wochschr.,* **69,** 648 (1939)
12. GLICK, D., *J. Biol. Chem.,* **134,** 617 (1940)
13. DAHL, O., *Arkiv Kemi Mineral. Geol., A,* **13,** 13 (1939)
14. EULER, H. v., AND BAUER, E., *Z. physiol. Chem.,* **261,** 125 (1939)
15. WILLSTÄTTER, R., AND ROHDEWALD, M., *Enzymologia,* **8,** 1 (1940)
16. COLOWICK, S. P., WELCH, M. S., AND CORI, C. F., *J. Biol. Chem.,* **133,** 359 (1940)
17. CORI, G. T., AND CORI, C. F., *J. Biol. Chem.,* **135,** 733 (1940)
18. SÜLLMANN, H., *Helv. Chim. Acta,* **23,** 606 (1940)
19. HANES, C. S., *Nature,* **145,** 348 (1940)
20. SOMOGYI, M., *J. Biol. Chem.,* **134,** 301 (1940)
21. MCCLURE, F. J., *U.S. Pub. Health Repts.,* **54,** 2165 (1939)
22. NINOMIYA, H., *J. Biochem. (Japan),* **31,** 421 (1940)
23. BALLOU, G. A., AND LUCK, J. M., *J. Biol. Chem.,* **135,** 111 (1940)
24. JANKE, A., AND HOLOTA, J., *Biochem. Z.,* **304,** 194 (1940)
25. HESTRIN, S., *Enzymologia,* **8,** 193 (1940)
26. BAUER, E., *Helv. Chim. Acta,* **22,** 1114 (1939)
27. FREIDENBERG, K., AND PLOETZ, T., *Z. physiol. Chem.,* **259,** 19 (1939)
28. MALAGUZZI-VALERI, C., *Arch. sci. biol. (Italy),* **25,** 261 (1939)
29. VAN ROBERTSON, B. W., ROPES, M. W., AND BAUER, W., *J. Biol. Chem.,* **133,** 261 (1940)
30. CLAUD, A., *Proc. Soc. Exptl. Biol. Med.,* **43,** 684 (1940)
31. FISHMAN, W. H., *J. Biol. Chem.,* **131,** 225 (1939)
32. MITUDA, H., *J. Agr. Chem. Soc. Japan,* **14,** 1485 (1938)
33. TADOKORO, T., AND TAKASUGI, H., *J. Chem. Soc. Japan,* **60,** 188 (1939)
34. STOTZ, E., *J. Biol. Chem.,* **133,** c (1940)
35. LOVETT-JANISON, P. L., AND NELSON, J. M., *J. Am. Chem. Soc.,* **62,** 1409 (1940)
36. RAMASARMA, G. B., DATTA, N. C., AND DOCTOR, N. S., *Enzymologia,* **8,** 108 (1940)
37. GREGG, D. C., AND NELSON, J. M., *J. Am. Chem. Soc.,* **62,** 2500 (1940)
38. GREGG, D. C., AND NELSON, J. M., *J. Am. Chem. Soc.,* **62,** 2506 (1940)
39. GREGG, D. C., AND MILLER, W. H., *J. Am. Chem. Soc.,* **62,** 1374 (1940)
40. KEILIN, D., AND MANN, T., *Nature,* **145,** 304 (1940)
41. HAUGE, S. M., *J. Biol. Chem.,* **108,** 331 (1935)

42. WILBUR, J. W., HILTON, J. H., AND HAUGE, S. M., *J. Dairy Sci.*, 18, 661 (1935)
43. FREY, C. N., SCHULTZ, A. S., AND LIGHT, R. F., *Ind. Eng. Chem.*, 28, 1254 (1936)
44. KIRSANOVA, V. A., *Biokhimiya*, 3, 191 (1938)
45. SUMNER, J. B., AND DOUNCE, A. L., *Enzymologia*, 7, 190 (1939)
46. TAUBER, H., *J. Am. Chem. Soc.*, 62, 2251 (1940)
47. SUMNER, J. B., AND SUMNER, R. J., *J. Biol. Chem.*, 134, 531 (1940)
48. LANG, K., AND ADICKES, F., *Z. physiol. Chem.*, 262, 123 (1940)
49. HOVE, E., ELVEHJEM, C. A., AND HART, E. B., *J. Biol. Chem.*, 134, 425 (1940)
50. WEIL, L., AND RUSSELL, M. A., *J. Biol. Chem.*, 136, 9 (1940)
51. TRAVIA, L., AND VERONESE, A., *Boll. soc. ital. biol. sper.*, 15, 467 (1940)
52. LOHMANN, K., AND SCHUSTER, P., *Biochem. Z.*, 294, 188 (1937)
53. TAUBER, H., *J. Am. Chem. Soc.*, 60, 730 (1938)
54. WEIJLARD, J., AND TAUBER, H., *J. Am. Chem. Soc.*, 60, 2263 (1938)
55. WEIL-MALHERBE, H., *Biochem. J.*, 34, 980 (1940)
56. OCHOA, S., *Nature*, 141, 831 (1938)
57. WEIL-MALHERBE, H., *Biochem. J.*, 33, 1997 (1939)
58. WESTENBRINK, H. G. K., AND GOUDSMIT, J., *Enzymologia*, 5, 307 (1938)
59. TAUBER, H., *Proc. Soc. Exptl. Biol. Med.*, 37, 541 (1937)
60. HENNESSY, D. J., AND CERECEDO, L. R., *J. Am. Chem. Soc.*, 61, 179 (1939)
61. LIPMANN, F., *Enzymologia*, 4, 65 (1937)
62. BARRON, E. S. G., AND LYMAN, C. M., *J. Biol. Chem.*, 127, 143 (1939)
63. BANGA, I., OCHOA, S., AND PETERS, R. A., *Biochem. J.*, 33, 1109 (1939)
64. SOBER, H. A., LIPTON, M. A., AND ELVEHJEM, C. A., *J. Biol. Chem.*, 134, 605 (1940)
65. LOHMANN, K., AND KOSSEL, A. J., *Naturwissenschaften*, 27, 595 (1939)
66. MELNICK, J. L., AND STERN, K. G., *Enzymologia*, 8, 129 (1940)
67. TAUBER, H., *J. Biol. Chem.*, 125, 191 (1938)
68. GREEN, D. E., HERBERT, D., AND SUBRAHMANYAN, V., *J. Biol. Chem.*, 135, 795 (1940)
69. GREENBERG, D., AND RINEHART, J. F., *Proc. Soc. Exptl. Biol. Med.*, 43, 495 (1940)
70. BUCHMAN, E. R., HEEGARD, E., AND BONNER, J., *Proc. Natl. Acad. Sci. U.S.*, 26, 561 (1940)
71. WESTENBRINK, H. G. K., AND VAN DORP, D. A., *Nature*, 145, 465 (1940)
72. LIPTON, M. A., AND ELVEHJEM, C. A., *J. Biol. Chem.*, 136, 637 (1940)
73. TAUBER, H. (Unpublished results)
74. LIPTON, M. A., AND ELVEHJEM, C. A., *Nature*, 145, 226 (1940)
75. LIEBKNECHT, W. L., *Biochem. Z.*, 303, 101 (1939)
76. STERN, K. G., AND MELNICK, J. L., *J. Biol. Chem.*, 135, 365 (1940)
77. WEIL-MALHERBE, H., *Nature*, 145, 106 (1940)
78. VAN GOOR, H., *Enzymologia*, 8, 113 (1940)
79. KEILIN, D., AND MANN, T., *Biochem. J.*, 34, 1163 (1940)
80. HOVE, E., ELVEHJEM, C. A., AND HART, E. B., *J. Biol. Chem.*, 136, 425 (1940)
81. MANN, T., AND KEILIN, D., *Nature*, 146, 164 (1940)

82. KIESE, M., AND HASTINGS, A. B., *J. Biol. Chem.,* **132,** 281 (1940)
83. DAVENPORT, H. W., *J. Physiol.,* **97,** 32 (1939)
84. KITAGAWA, M., *J. Agr. Chem. Soc. Japan,* **15,** 267 (1939)
85. RICHARDS, M. M., AND HELLERMAN, L., *J. Biol. Chem.,* **134,** 237 (1940)
86. FROMAGEOT, C., AND MOUBASHER, R., *Enzymologia,* **2,** 231 (1938); FROMAGEOT, C., WOOKEY, E., AND CHAIX, P., *Compt. rend.,* **209,** 1019 (1939)
87. JAQUES, L. B., *J. Biol. Chem.,* **133,** 445 (1940)
88. KUNITZ, M., *J. Gen. Physiol.,* **24,** 15 (1940)
89. WEAST, C. A., AND MACKINNEY, G., *J. Biol. Chem.,* **133,** 551 (1940)
90. SUMNER, J. B., DOUNCE, A. L., AND FRAMPTON, V. L., *J. Biol. Chem.,* **136,** 343 (1940)
91. WERLE, E., *Biochem. Z.,* **304,** 201 (1940)
92. TORISU, T., *Japan. J. Med. Sci., IV, Pharmacol.,* **12,** 1 (1939)
93. WERLE, E., AND BODEN, W., *Biochem. Z.,* **304,** 371 (1940)
94. COHEN, P. P., *J. Biol. Chem.,* **136,** 565 (1940)
95. COHEN, P. P., *J. Biol. Chem.,* **136,** 585 (1940)

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CHEMISTRY OF THE CARBOHYDRATES
AND GLYCOSIDES

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Fifty years have elapsed since Emil Fischer established by synthesis the general structure of the sugars. To some extent carbohydrate chemistry might be said since then to have divided dichotomously into a monosaccharide branch on the one hand, and a polysaccharide branch on the other. Towards the center their branches are intermingled and are becoming more so as the more complex polysaccharides come under extensive study. The application of what might be called monosaccharide chemistry to the study of the single-component polysaccharides was until recently limited to the identification of the constituent sugar and the determination of its configuration, usually not a particularly difficult matter. With the development of such procedures as chemical end-group assays by the separation of methylated hydrolysis products, or the identification of residual aldobionic acids by comparison with synthetic acids of known configuration, the wealth of information as to the reactions, derivatives, and structure of the simple sugars is finding valuable use in solving problems concerning the molecular form of the homogeneous single-component polysaccharides, and in formulating the constitution of the nonhomogeneous polysaccharides. The author of the corresponding chapter in Volume IX of this *Review* was "impressed with the realization that the structures and configurations of the simple sugars and their derivatives have been proved." It would be quite inaccurate yet to make any such broad claim for the polysaccharides which are far less reactive than the simple sugars, and which provide problems of molecular configuration that cannot be completely solved by the use of ordinary chemical tools. The differences in properties between starch and cellulose are not fully explained by the statement that the constituent units in the former are α-glucopyranose and in the latter β-glucopyranose, both linked in the 1,4-positions.

The formation of polysaccharides is apparently a very specialized form of polymerization. Many of the properties of polysaccharides are more directly the result of a certain molecular architecture than a

function of the constituent sugar units. Comparatively mild treatments may bring about extensive chain shortening without producing any easily detectable change in chemical properties or reactivity. Moreover, many of the polysaccharides do not exist in a free condition nor in a form in which they may be easily separated from associated tissue constituents, and one of the serious problems confronting the polysaccharide chemist is the determination of the nature of the changes that may have been effected in the process of isolation of his product. Cellulose in the form of the cotton fiber, and starch in cereal grains or roots, are two polysaccharides which may most readily be obtained in a pure condition by mild means, and it is no accident that studies on molecular structure and architecture have gone further on such materials than on polysaccharides, such as pectin and the hemicelluloses, that can be obtained only by some more drastic extraction process. The situation is rendered more complicated by the fact that the same polysaccharide in different species of plants may not have identically the same molecular architecture, even though the monosaccharide bricks and the general pattern in which they are laid may be the same. Such differences will probably be found to be the rule rather than the exception.

It would be incorrect however to give the impression that in the solution of these problems of molecular architecture conventional carbohydrate methods can make little contribution, or that physical methods only are likely to give the correct answers. Indeed the chemical methods of attack, mostly making use of some properties of the terminal units in the molecule to deduce molecular size, have recently received much attention, and have been admirably reviewed by Haworth (1). These chemical methods have given results which at first sight appear to differ from the conclusions drawn from physical measurements. However the differences have arisen largely because of the primary assumption that has been made, that polysaccharide molecules should be represented as simple uniform chains. Husemann (2) and Hess et al. (3, 4) have recently discussed the significance of the end-group determination in the chemistry of the polysaccharides, the latter especially as applied to cellulose.

Starch appears to have a greater constancy of structure than cellulose. Starch molecules, irrespective of botanical source, have a branched form built of basal chains or repeating units each composed of from twenty-four to thirty glucose residues. These repeating units are presumed to be joined by a primary valence linkage, the precise

position of which is in doubt, though Freudenberg (5) favors linkage through C_6. In contrast to the branched structure of starch, cellulose is presumed to be a molecular aggregate of chains, the minimum length of each of which is not less than two hundred glucose units. If, however, methylation is carried out not in air but in an inert gas, the end-group method indicates a much greater chain length. This has been explained by the assumption that the cellulose molecule may consist of parallel chains linked head to tail, and stabilized by cross linkages or intermediate bonds of primary valence, perhaps every twenty-five to thirty glucose units. Detailed review at this stage would be premature, but this will be sufficient to show that the older concepts of the molecular structure of the polysaccharides are being revised.

The position in polysaccharide chemistry today is that there are only a few polysaccharides, of which the nature and configuration of the component units are fully accepted and about which there is some definite information concerning molecular structure and form. Of the remaining polysaccharides either the structural arrangement of the component units has not yet been determined, or the constituent groups have not been identified, or the linkages between the constituent groups have not yet been confidently established. Attention will be directed in this report primarily to representatives of the latter groups.

Water-soluble polysaccharides.—The water-soluble polysaccharides are usually simple hexosans, and are probably of relatively small molecular dimensions. The wide-spread existence of fructosans or levans in many plants has long been recognized but few representatives of this type, apart from inulin, have been subjected to the newer procedures of constitutional carbohydrate chemistry nor has much attention been given to their possible roles in plant metabolism. A comprehensive review of the distribution of fructosans in the monocotyledons was published by Archbold (6). In many grasses the fructosan content may be considerable at about the time of heading. The subsequent rapid growth appears to be at the expense of this polysaccharide (7, 8) which may therefore be considered as a transitory reserve. Smaller quantities are found in cereals, the main region of accumulation being the stem. Conditions which tend to promote sugar storage seem to result also in the accumulation of fructosan. High nitrogen fertilization markedly reduces the fructosan content. The fructosans of barley are slowly hydrolyzed by the invertase of

yeast (9) and this seems to be true also, in varying degrees, of fructosans from other sources. Hydrolysis of the methylated fructosan from barley leaves (10) yielded 1,3,4-trimethylfructofuranose and led to the conclusion that the linkage is through carbon atoms 2 and 6, differing in this from inulin in which the linkage is 1,2. The molecular structure is believed to consist of a straight chain of about ten to twelve hexose units.

The fructosan of the bulbs of *Allium* (scorodose) similarly yields 1,3,4-trimethylfructofuranose and 1,3,4,6-tetramethylmethylfructoside (11). That the fructosans of plants may not all be identical is indicated by the work of Schlubach *et al.* (12). Secalin, the fructosan from rye, is said to yield a mixture of methylated hydrolysis products in proportions which point to a tetrafructose anhydride structure (13). However, the 2,6-anhydrofructose compounds from plant sources would appear to be similar to the bacterial levans formed by aerobic sporeformers of the *mesentericus-subtilis* type. Preparations from certain soil actinomycetes gave trimethyl derivatives apparently identical to those obtained from plant levans, but triacetates of different rotation and melting point (14). Lyne, Peat & Stacey (15) have ascribed such anomalies to incomplete acetylation. They described the conditions for acetylation of the levans formed by *Bact. pruni* and *Bact. prunicola* which also consist of ten to twelve contiguous fructofuranose units linked through the 2,6-positions.

Evidence of the presence of water-soluble dextrans or glucosans in plant tissues has frequently been obtained in detailed sugar analyses. From barley roots Hassid (16) has isolated 0.4 per cent glucosan, which is readily hydrolyzed with acid but is unaffected by invertase or β-amylase. Methylation and subsequent hydrolysis gave 2,3,4-trimethyl-β-methylglucoside, pointing to the probability of 1,6-linkage between glucopyranose units. Similar polysaccharides, perhaps not strictly water-soluble, are formed by *Betacoccus* (*Leuconostoc*) *arabinosaceus* and closely related species. Peat, Schlüchterer & Stacey (17) found methylated bacterial dextran to exhibit considerable resistance to hydrolysis, but obtained 90 per cent 2,3,4-trimethylglucose, 10 per cent of a mixture of dimethylglucoses, and 0.23 per cent tetramethylglucose, and deduced therefrom a 1,6-α-*d*-glucopyranose structure. The chemical evidence from end-group analysis indicated a chain length of about 550 glucose units whereas osmotic pressure measurements gave a value corresponding to 200 units. Like discrepancies have been found in other dextran preparations apparently

of similar constitution. Hassid & Barker (18) obtained a molecular weight of 11,700 by the Staudinger viscosity procedure as opposed to a value of 2,600 ± 50 by the sedimentation equilibrium method in the ultracentrifuge. The dextrans produced by *Betabacterium vermiforme* seemed by osmotic methods to have a molecular size corresponding to a chain length of about 500 glucose units, but in this case chemical end-group determinations indicated much shorter chains, consisting only of about twenty-five units (19).

From certain coniferous woods, particularly from that of the larch, a water-soluble polysaccharide of somewhat different character can be extracted, often in considerable amount. The products from different sources seem relatively uniform in properties and gross composition. They may properly be described as arabogalactans in which the ratio of arabinose units to galactose units is about one to six. Their chemical identity and homogeneity is not established however; indeed a study of the acetates, propionates, and benzoates of preparations from Eastern, Western, and European larches makes it seem improbable that only a single polysaccharide is present (20). The propionates of western larch polysaccharide were separated into five fractions of the same acyl content, but with variable rotation, reducing value, and araban content. The viscosities of arabogalactan solutions in water are directly proportional to the concentration up to about 6 per cent, but beyond this increasing deviations from linearity occur. Owens (21) interprets these observations as being indicative of a spherical molecular form. Husemann (22) is of the opinion that the arabogalactan molecule is highly branched, like starch.

Cell-wall polysaccharides.—Cellulose is, of course, the major polysaccharide of the plant cell wall, but the cellulosic fabric of the wall is infiltered and encrusted with other polysaccharides falling within the ill-defined group of hemicelluloses. The study of these associated polysaccharides presents many difficulties because they possess few properties that can be used in separation, and also because satisfactory criteria of purity have not been found. In mature tissues lignin also is present, and until recently delignification could not be accomplished without some concurrent attack on, or removal of, polysaccharide material with which it may be actually combined. The so-called *Skelettsubstanzen* obtained by prolonged treatment of lignified tissues with chlorine dioxide was said to represent the polysaccharide framework of the cell wall (23). Either as a qualitative or especially as a quantitative method, however, this procedure was

unsatisfactory, because it was slow and lacked any clearly determinable end point. A similar product, called holocellulose, was prepared by alternate gaseous chlorination and extraction with a mixture of equal parts of alcohol and pyridine (24). For the latter reagents 95 per cent ethanol containing 3 per cent monoethanolamine was later substituted (25). The chlorine resolves the lignin-hemicellulose linkage and forms chlorinated and oxidized derivatives of lignin which are extracted by a nonaqueous solvent, thereby preventing solution of the hemicelluloses. A standard procedure has been proposed (26) but many variations are possible. After chlorination, and in place of the solvent extraction, treatment with sodium or calcium hydroxide just sufficient to make the solution alkaline has been substituted (27, 28, 29, 30). Others have employed chlorine water followed by 0.1 per cent sodium hydroxide in 95 per cent alcohol, or various concentrations of ammonia in 84 per cent alcohol (31, 32).

The examination and hydrolysis of holocellulose preparations has thrown considerable light on the distribution of methoxyl, acetyl, and uronic groups in wood. All the acetyl and part of the methoxyl groups of maple were found to be associated with the holocellulose fraction and not with lignin, a portion of both being attached to the cellulose component (24). If the more easily hydrolyzed carbohydrates be removed from holocellulose, a cellulosic fraction remains which, under certain empirical conditions, may be comparable in yield with the Cross and Bevan cellulose, ordinarily obtained by alternate treatment with chlorine and neutral aqueous sulphite. The two residues, however, are not chemically identical (33). The viscosities of holocellulose preparations may be greater than those of Cross and Bevan cellulose despite the presence of appreciable amounts of hemicelluloses. The cellulose chains are apparently shortened in the Cross and Bevan cellulose procedure or more extensively by dilute acid hydrolysis of holocellulose (34).

Extensive delignification with concurrent removal of some hemicellulose may be readily accomplished by direct extraction of the tissue with monoethanolamine for five hours at 170° (35). To secure complete delignification the residue must then be chlorinated, either by chlorine gas or chlorine water, and extracted with hot aqueous 3 per cent sodium sulphite. This residue is said to be quantitatively and qualitatively comparable to Cross and Bevan cellulose, though in view of the high temperature involved this is surprising. The ethanolamine procedure compares favorably in ease of operation with other

methods of cellulose determination (36), though the length of time necessary for extensive delignification seems to vary rather widely.

The holocellulose procedures may be employed in microscopic studies of the distribution of the various cell-wall constituents (37). The delignified tissues retain the structural arrangement of the fibers and the ray cells. Ethanolamine in the cold slowly removes all secondary cell-wall lignin, but does not appreciably attack that of the middle lamella (35). Preston & Allsopp (38) have studied by x-ray analysis the effect of the removal of lignin, hemicelluloses, and cellulosans from the cell wall of several woods and the coir fiber. The removal of cellulosans has little effect, but delignification is accompanied by a decrease in the angular dispersion of the cellulose chains, and, in coir, a slight change in their orientation. The inference is that lignin occurs in the intermicellar spaces and not within the cellulose micelle. The arrangement of the cellulose crystallites in ray cells of white oak has been determined (39). They appear to deviate about 30° from the vertical crosswise direction in the ray cells, which is in keeping with microscopical observation and swelling data. Three good reviews of recent studies on the structure and microcomposition of cellulosic membranes and fibers have appeared (40, 41, 42).

The cotton hair lends itself well to the investigation of the development of a cellulosic wall. It now appears that in the early stage, when the cell is elongating, the cellulosic deposition is of a different character than that which occurs later in the secondary thickening (43, 44). Hess *et al.* (45) have discussed the formation of cellulose in the cell wall, and Mark (46) has summarized the implications of recent x-ray diffraction findings on the structure of cellulose, particularly with respect to the intermicellar spaces. Microscopical and microchemical studies on the chemistry of the plant cell wall have been continued by Harlow (47), who by the application of various solvents has attempted to locate the position of lignin, cellulose, and other cell-wall components. In delignification the layer most resistant appears to be the primary wall. Fully delignified wood sections do not macerate in the sense that each cell separates from its neighbors. Microphotographs of high quality and resolution have been obtained by infrared photography of wood sections stained with neocyanine (48).

Hemicelluloses.—The chemistry of the hemicelluloses is still chaotic, and indeed the very term is unfortunate. Under this heading are generally included those cell-wall polysaccharides extractable by dilute alkalis, either hot or cold, and hydrolyzable by hot dilute acids

to their constituent sugars or sugar acids. The group includes not only all short-chain hexosan and pentosan (cellulosans) associated and oriented with the cellulose chains, rightly to be considered a part of the cellulosic aggregate, but also the encrusting amorphous polysaccharides which may conceivably be in part linked to lignin and which seem invariably to contain uronic units (polyuronide hemicelluloses). The distinction between these two classes is not sharp, and is based primarily on the separation effected in the Cross and Bevan cellulose procedure. Pretreatments, or variations in the procedure may result in a different separation; the distinction therefore has been criticized as being quite empirical. Investigations of the hemicelluloses have usually been mainly directed towards the polyuronide group, but in most cases there is little or no assurance that the preparations do not contain some component from the cellulose. Preece (49) has examined the influence of alkaline treatments and pretreatments on the isolation of hemicelluloses, and has shown that hot solutions of sodium hydroxide in water, or in alcohol as sometimes employed, have a degradative effect indicated by significant reduction in the furfuraldehyde yields. Pretreatment of the wood of *Eucalyptus regnans* with hot alkali as dilute as 0.16 per cent has been found to alter somewhat the partition of the hemicelluloses effected by the Cross and Bevan cellulose procedure, the influence of the alkali being to increase slightly the furfuraldehyde-yielding components in the cellulose aggregate (32).

The hemicelluloses of wood have been more thoroughly investigated than those from other sources. Continuing her very extensive studies on the hemicelluloses of English oak, O'Dwyer (50) showed that the hemicellulose-A fraction of sapwood (precipitated from the alkaline extract by simple acidification) contains glucose units that may be removed by treatment with taka-diastase or long digestion with water at 100°. No glucose occurred in the preparation from heartwood. The latter, or the glucose-free residue from the former, on prolonged treatment with taka-diastase yielded three parts of xylose and two parts of a soluble polysaccharide, the component units of which were monomethoxyhexuronic acid and xylose in the proportions of one to six. On hydrolysis a resistant aldobionic acid consisting of xylose-methoxyhexuronic acid was obtained. The position of the methoxy group on the hexuronic acid has not been determined. It is markedly resistant to both alkaline and acid treatments.

Similar studies of the hemicellulose-B fraction (precipitated from

the alkaline extract only by acid-alcohol) from the same sources left little doubt that this fraction also contains a basic recurring structural unit consisting of six anhydroxylose units and one methoxyhexuronic unit (51). Again the sapwood preparations contained considerable amounts of glucose and were dextrorotatory, in contrast to heartwood preparations with $[\alpha]_D$ *circa* —53°. It appears that in the transformation of sapwood to heartwood, the main constitutional change that occurs in either the A or the B fraction consists in the removal of anhydroglucose units. These observations indicate to some degree the extent and nature of the separation effected when the alkali-soluble extract is divided into two fractions, the first by acidification and the second by the addition of alcohol thereto. The differences in the optical rotations of the fractions are such as to suggest that they do represent separate polysaccharides or groups. The alcohol-precipitated fractions were also of higher uronic content, but higher uronic content might be associated with a shorter chain length or smaller molecular size, and might not necessarily be of itself the prime reason for the difference. If the structure of these hemicelluloses is one of relatively simple chain molecules, such as six or twelve or more pentose groups to one hexuronic group, then separation on a physical basis might be accompanied by clear differences in the proportion of the various components. On the other hand, if their structure is one of large chain molecules, straight or branched, with varying numbers of recurrent units, such as ten, twenty, or thirty repetitions of six or twelve pentose groups linked to one hexuronic group, then physical separation would not be accompanied by any significant differences in composition. If, as is conceivable, both possibilities occur together, other methods of separation will have to be devised.

Several woods were studied by Anderson *et al.* (52) in an attempt to ascertain whether the polyuronide hemicelluloses of various hardwoods are identical. Their results resemble those of O'Dwyer in many particulars though a more exhaustive extraction was given, and a more elaborate scheme of fractionation followed. The least soluble fractions had the lowest uronic contents. A blue color was given with iodine only in the most soluble fraction from two of the woods, lemonwood and black locust sapwood. The glucose groups responsible for this coloration could not be removed even on prolonged treatment with taka-diastase. Prolonged hydrolysis with 4 per cent sulphuric acid yielded two acids, the analyses of which corresponded closely to the requirements of one methoxyhexuronic unit linked respectively to

one, and two, anhydroxylose units. Most preparations could be almost completely accounted for in terms of methoxyhexuronic acid and xylose. The proportions in the least soluble fractions were from seventeen to nineteen xylose units per hexuronic group, and in the more soluble fractions mostly from ten to twelve xylose units, though figures from eight to fifteen were obtained. They conclude that the polyuronide hemicelluloses of hardwoods are similar in general type, but that in some woods transition polysaccharides, relating wood starch to hemicelluloses, may be present. In these, glucose units are attached to the xylan chain with the result that the preparations give a strong iodine color and are less markedly levorotatory.

Preparations from lignified tissues other than woods appear to be similarly composed. Cottonseed hulls yielded fractions in which from ten to sixteen xylose groups were present per d-glucuronic unit (53). By repeated fractionation Krznarish (54) obtained a series of preparations from oat hulls, certain of which were of such low uronic content that they might be considered as true pentosans. The more soluble preparations however were of a polyuronide character and apparently of the same general xylan-uronide nature. Weihe & Phillips (55) found that the easily extractable hemicelluloses of wheat straw were mostly of the B type, precipitable only on the addition of a solvent. The uronic content of this fraction was 5.73 per cent, and the remainder of the molecule was not fully accounted for by the presence of pentose sugars. d-Xylose and l-arabinose were identified among the products of acid hydrolysis and the relative proportions of hexuronic acid, arabinose, and xylose were 1, 0.9, and 23.

Few recent studies have been made of the polyuronide hemicelluloses of younger, less lignified tissues. In general the component sugars have been reported to be of the arabinose-galactose series, the hexose usually being present in considerable amount. This is in contrast to those from lignified tissues that seem to be mainly of the xylose-glucose series with the pentose always predominant. Alfalfa hay, which perhaps is intermediate in character, has given preparations typical of a lignified source, the main fraction of which contained 12.13 per cent uronic anhydride, probably methoxyhexuronic acid (2.27 per cent methoxyl), and 77.3 per cent pentosan, mainly d-xylose but also including a little l-arabinose (56).

Almost all investigators in this field have used 4 per cent sodium hydroxide, either cold or hot, for the extraction of the plant material, with two results: that the extract frequently contains lignin which

must subsequently be removed from the preparations; and that a mixture of polyuronide hemicelluloses and cellulosans must inevitably be obtained. There is no evidence that satisfactory preferential extraction of the former can be obtained. Polyuronide hemicelluloses, however, may be partially or completely linked with lignin *in situ* (57), and if some treatment capable of resolving this linkage be applied, subsequent extraction of these polysaccharides may be achieved by means much less drastic than ordinarily employed. The so-called "holocellulose" procedures referred to on page 70, which accomplish complete delignification without attack on the carbohydrates, provide admirable starting material for the extraction of hemicelluloses. Using holocellulose from sugar maple, Mitchell & Ritter (58) obtained four major hemicellulose fractions by successive treatment with hot water for one hour, with 2 per cent sodium carbonate cold for forty-eight hours, with 4 per cent sodium hydroxide cold, and with 10 per cent sodium hydroxide hot for one hour. The total loss sustained by the holocellulose as a result of these treatments amounted to about 25 per cent of the wood. Two preparations, IA and IB, were obtained from the hot water extract, the former on acidification and the latter only after addition of alcohol. The sodium hydroxide extracts undoubtedly contained nonuronide material of a cellulosan nature, and the analyses of these fractions were not unlike those of other preparations from wood, namely eight to ten xylose groups per methoxyhexuronic group. The preparations obtained by the milder methods, IA, IB, and II (sodium carbonate) were particularly interesting since they may represent a portion of the true cell-wall polyuronides unadmixed with less soluble polyuronides or cellulosans. These had respectively 17.1, 15.8, and 22.8 per cent uronic anhydride and a proportion of xylose groups to uronic acid in the neighborhood of three to one. In addition the water-soluble fractions contained nearly 25 per cent hexosan and 9.3 per cent acetyl. The latter may prove to be a general constituent, hitherto undetected because of the alkali treatment ordinarily given in extraction. An attempt was made to determine the minimum molecular weight of these fractions by hypoiodite determination of the end-group reducing value. The water-soluble fractions gave figures approximating ten and twenty sugar units respectively.

Little information is available as to enzymes capable of hydrolyzing the hemicelluloses. One of the components of taka-diastase brought about slow digestion of preparations from oak wood with

slow liberation of glucose and xylose units (50). The kinetics of breakdown of "xylan" have been investigated (59). The hemicelluloses of linden wood *in situ* are not in a form easily hydrolyzable by enzyme preparations from the digestive juices of *Helix pomatia* (60), but when extracted by means of a solution composed of copper oxide dissolved in ethylenediamine and precipitated, these hemicelluloses were extensively digested. A portion of the hemicelluloses of young living tissue (elder tree pith, and araucaria pith and bark) were readily digested by enzymes from the same source, but the residues were not pentosan-free and contained lignin and carbohydrate in simple equivalent proportions (61).

In ordinary pulping processes the hemicelluloses are removed concurrently with the lignin and discarded. Newer delignification processes may permit all or part of this group to be isolated along with the cellulose and their presence affects some of the physical properties of the pulp, particularly swelling (62, 63).

Pectin.—Pectin for many years has been known to consist primarily of galacturonic acid. Preparations considered to be of reasonable purity were usually found to contain 65 to 80 per cent urone and 9 to 11 per cent methoxyl. Some arabinose and galactose seemed invariably to be present, though the proportions varied, and could be reduced by mild acid treatment. The very extensive researches of Ehrlich (118) led him to suggest that pectin could be formulated in a general way as a calcium salt of a methoxylated tetragalacturonic acid ring to which galactose, arabinose, and occasionally other sugars are loosely attached. Associated with the pectin were ordinarily found small quantities of a tetra-araban, which Ehrlich believed to be formed by simple decarboxylation of the tetragalacturonide. Others put forward the view that pectin could be represented by a six-membered ring containing the same constituent units. Sometime later it was suggested that the nucleus of the pectin molecule is octagalacturonic acid, since preparations of high viscosity and jellying ability could be obtained, the arabinose and galactose contents of which were far below the proportions called for by the other proposals (64). These sugars were, however, still considered to be a part of the molecule, the physical properties of which were held to depend on the degree of polymerization of the molecular aggregate. Such a concept became increasingly improbable as information concerning the natures of other polysaccharide macromolecules accumulated. It now appears that the essential part of the pectin complex consists of a long chain of galacturonide

units, the arabinose and galactose invariably found in ordinary preparations being derived from associated araban and galactan. Preparations of high uronide content (*circa* 95 per cent) can be obtained by treatment of isolated pectin with dilute acid and dilute alkali, and correspond to the "pectolic acid" described by Ehrlich. With dry methanol and hydrogen chloride a water-soluble α-methylpolygalacturonide ester was obtained, the methyl ester groups of which could be removed by treatment with dilute alkali (65). The glycosidic methoxyl group then could be used as indicative of chain length. By this means it was concluded that the minimum length of the polygalacturonide chain in citrus pectin must be eight to ten units and the probable length much greater.

An elongated chain molecular structure for pectin was deduced as a result of x-ray studies on dinitropectic acid (66, 67). Neither osmotic nor viscosity methods for the determination of molecular weight are directly applicable to pectin, but esterification with nitro, acetyl, or formyl groups gives products that are amenable to such measurements. High molecular weights, 30,000 to 100,000, were obtained, varying with the method of preparation (68). That these high values are really due to macromolecules was indicated by the fact that the relation between specific viscosity and temperature was of the same order of magnitude as for other macromolecular substances, such as cellulose esters.

The chain molecules of the pectin esters were considered to be less extended than the cellulose molecule, possibly because of the presence of strongly polar carboxylate groups. Schneider & Fritschi (68) by further examination of nitropectin preparations arrived at the conclusion that Ehrlich's formula was completely untenable, and that the arabinose and galactose ordinarily found in pectin preparations were due to the presence of accompanying araban and galactan not linked in any way to the long-chain, partially esterified polygalacturonide that essentially formed the pectin molecule.

Several procedures for the preparation of the polygalacturonide free from associated araban or galactan have been devised. The araban of peanut pectin was removed by cold extraction with 70 per cent alcohol for many weeks, and purified by acetylation and deacetylation (69). Hydrolysis with 0.05 N HCl yielded *l*-arabinose in almost theoretical yield for a pure araban. The susceptibility to hydrolysis made it probable that the arabinose residues were exclusively and uniformly of a furanose type. Alcoholic extraction of apple pectin,

however, gave a mixture of araban and galactan that could only be separated after methylation (70). The araban may also be removed from the pectin complex or from the araban-galactan mixture by preferential hydrolysis with 0.05 N acid, in which the galactan remains unaffected. Galactan was removed from the pectic acid by repeated solution in alkali and reprecipitation of the latter as a calcium salt. Under alkaline conditions the araban proved to be more stable than the pectic acid. Methylated araban was obtained by heating apple pectin with methyl sulphate and sodium hydroxide, as a result of which the pectic acid was largely destroyed and the galactan hardly affected. After completion of the methylation and purification, a fully methylated araban was obtained with properties similar to the methylated araban from the peanut. On hydrolysis an equimolecular mixture of three different methylated arabinoses, 2,3,5-trimethyl-*l*-arabofuranose, 2,3-dimethyl-*l*-arabinose, and 3-methyl-*l*-arabinose, were obtained, the identity of the first two being established with assurance. Hirst & Jones (70) concluded that all the arabinose units in these preparations were furanose in structure and probably of the α-configuration. The chain linkage would be through hydroxyl groups on C_1 and C_5, but the chain must be branched since in some residues C_2 was also involved. The araban from citrus pectin was obtained in impure condition by extraction with hot 70 per cent alcohol. After purification by acetylation and deacetylation a product was obtained apparently identical in structure with those just mentioned (71). It is to be noted that an araban exclusively composed of arabofuranose units could not be formed by the decarboxylation of a polygalacturonide unless the units therein were also of the furanose type.

The structure of citrus polygalacturonide freed from araban and galactan has been studied by methylation and hydrolysis of the methyl ester of the methylated pectic acid obtained (72, 73). The main product was the methyl ester of 2,3-dimethylmethylgalacturonoside, the structure of which was determined by oxidation to 2,3-dimethyl mucic acid which formed a crystalline γ-lactone methyl ester, and by methylation to give the methyl ester of 2,3,5-trimethyl-β-methylgalacturonoside, separately obtained by synthesis (74).

It is therefore certain that the galacturonic acid residues in pectic acid are linked in positions other than C_2 or C_3. This condition may be satisfied in one of two ways. Either the uronic units have a furanose form and are joined by 1,5 linkages, or a pyranose form with 1,4-glycosidic linkages. At first sight the evidence would seem to

favor the former, since a methylated derivative with furanose structure was the main product isolated in methylation studies, but it seems that 2,3-dimethylgalacturonic acid has a tendency preferentially to form a methyl furanoside when treated with acid methyl alcohol. Because of the high positive rotation of pectic acid and its fully methylated derivative ($[\alpha]_D > +200°$) and the stability of both toward hydrolytic agents Luckett & Smith (73) consider that the 1,4-α-glycosidic structure of pyranose units is the more probable. A similar conclusion has been arrived at for strawberry and citrus pectins (75) though the details have not yet been published. Thus it appears that pectic acid is constitutionally similar not to cellulose but to starch. One property common to both, and not manifest by a 1,4-β-glycoside such as cellulose is an extreme susceptibility to degradation by hot dilute alkali. The furanose alternative is preferred by Levene, Meyer & Kuna (76) as a result of a study of the rate of hydrolysis of the methyl ester of a methylated pectic acid. Luckett & Smith (73) comment on the fact that the methyl ester of trimethylmethylgalacturonoside was not detected among the hydrolysis products of methylated pectic acid, which would point either to an immensely long chain or else some form of loop structure in which the end groups are not free. Osmotic pressure, viscosity, and melting point depression measurements, however, indicated a molecular size of about thirteen units, which is of the same order as found by Link *et al.* (65).

The high viscosity of pectin in solution has been regarded by analogy as being due to the presence of long chain polygalacturonide molecules, and any reduction in viscosity has been attributed to scission and shortening of these chains. Additional evidence that pectin molecules may possess some double or branched structure, similar to that of starch, has been provided by Kertesz (77) in a study of viscosity changes accompanying enzymic degradation. A structure which may be represented by $[(G)_m]_n$ is postulated, in which $(G)_m$ is a polygalacturonide, the n units of which form a secondary aggregate which is mainly responsible for the viscosity of pectin. The viscosity of enzymically hydrolyzed pectin decreased far more rapidly than the increase in reducing power which should accompany simple chain scission. Moreover viscosity could be extensively and permanently reduced by heating at pH 3.2 without any increase in reducing power or decrease in methoxyl content. If the galacturonide chains are relatively short, as is possible, the low reducing power of pectin preparations remains to be accounted for, but this is a problem similar to that

encountered with starch, and perhaps may be explained by some sort of loop structure.

Pectin is readily hydrolyzed by pectinase preparation from fungi, usually from species of *Penicillium* or *Aspergillus*. This fact has been made use of in the preparation of *d*-galacturonic acid in a pure condition and in good yield (78, 79). Taka-diastase preparations usually contain pectin enzyme components (80). Pectin isolated from flax straw that has been attacked by rust organisms is substantially degraded (81).

One of the most important and widely investigated properties of pectin is its capacity to form rigid jellies with the appropriate quantities of sugar and acid. Many attempts have been made to relate the jelly-forming capacity of pectins to some one factor, and while certain relationships between the chemistry and the jellying power of pectins do exist, no simple explanation has yet been given. This subject has been authoritatively reviewed by Hinton (82), who divides the factors into two groups, (*a*) the inherent quality of the pectin, which varies with the source and the degree of modification that may have been effected in extraction; and (*b*) circumstances in the actual jellying process, such as pH conditions and the presence of salts. The magnitude of the jellying power governed by the first group of factors is probably dependent in some way on the molecular size or form of the pectin molecule, whereas the degree to which this power is manifest in actual jellies depends on the properties of the nonesterified carboxyl groups in the molecule and their relation with anion and cation constituents of the jellying mixture.

The viscosity of pectin solutions is frequently employed as a measure of potential jellying power and within certain limits may be satisfactorily so used. Baker & Goodwin (83) showed that dilute pectin solutions when adjusted to pH 2.6 give viscosity values which satisfy the logarithmic relationship of Arrhenius ($\log \eta_r = KC$), and that below pH 2.75 viscosity and jellying power are directly correlated. Little correlation could be found between chemical composition (mainly urone content) and viscosity or jelly strength of pectin preparations from a variety of sources (84). Some previous investigators had claimed that a relationship existed between the degree of esterification of pectin and jellying capacity, but no such relationship could be confirmed. It is probable that demethoxylation under some conditions accompanies disaggregation of the molecule, and that the loss of jellying power caused by the latter might be ascribed to the former, which

is a more readily detectable change. Some physicochemical aspects of the phenomenon of jelly formation have been examined by Hinton (85) who pointed out that pectin should probably be regarded as a complex mixture of carboxylic acids of variable composition which cannot be separated into homogeneous molecular components but which can be treated as a statistical population. The pectin molecule has the peculiarity of possessing a variable equivalent weight or degree of acidity depending on the extent of esterification. The carboxyl groups when entirely in the free condition may be expected to be dissociated only to a slight degree with the result that the pectin is little ionized, and coagulation or jellying can take place. If alkali is added the number of dissociated groups increases through salt formation with the result that there is a progressive tendency to inhibit jellying. The distribution of dissociated acid groups along the molecular chain must affect the number necessary to bring about effective ionization. When extensive de-esterification takes place the acid groups in the chain would be more numerous, and it would then be unnecessary for every group to be dissociated to inhibit jellying. The apparent dissociation "constants" of pectins from several sources have been determined experimentally and have been shown to diminish with increasing degree of neutralization with alkali. The strengths of jellies from one pectin preparation were found to be proportional to the excess of nonionized pectin above the limit of its solubility.

Alginic acid.—One of the most interesting of the polyuronides is the substance alginic acid, or algin, which occurs in considerable amount in many marine algae, and which may readily be extracted by treatment with cold dilute sodium carbonate. It has been established for some time that alginic acid is a polymannuronide in which the carboxyl groups are free and the aldehydic groups shielded by linkage. The identification of *d*-mannuronic acid was accomplished by characterization of the alkaloid salts, and comparisons with derivatives of the synthesized acid obtained by reduction of *d*-mannosaccharic dilactone (86). The alginic acid from two additional algae, *Turbinaria ornata* (87) and *Costaria turneri* (88) has been found to conform to this pattern. Variations in the neutralization equivalents of alginic acid preparations, however, led to the suggestion that certain of the mannuronic units in the polysaccharide might be in the lactone form. Lucas & Stewart (89) studied the esterification of this polyuronide on the ground that since lactone formation would reduce to one the number of hydroxyl groups per unit available for ester

formation, the degree of esterification would throw light on the extent of lactonization. On nitration with a mixture of nitric and sulphuric acids a variable number of nitrate groups from 0.49 to 1.2 per uronic unit could be introduced, and the failure to obtain higher nitration products was ascribed to the lactonization of the acid units either as a result of previous drying or because of the strong acid treatment. Full methylation with methyl sulphate could not be achieved, but diazomethane was more satisfactory. Methylation was not restricted to the carboxyl groups. Hirst, Jones & Jones (90) obtained a degraded alginic acid by boiling with 10 per cent hydrogen chloride in methanol from which, on methylation and hydrolysis, was obtained the methyl ester of 2,3-dimethyl-d-mannuronide. Further hydrolysis yielded 2,3-dimethyl-d-mannuronic acid which on oxidation with bromine formed 2,3-dimethyl-d-mannosaccharic acid. More extensive oxidation with periodic acid yielded glyoxylic acid and the semialdehyde of *meso*dimethoxysuccinic acid, and led to the conclusion that the major part of the alginic molecule is composed of β-d-mannuronic residues. The glycosidic linkage must be either 1,4 (pyranose ring) or 1,5 (furanose ring) since the methyl groups were on carbon atoms 2 and 3, and because of the extreme stability of the polyuronide and its high levorotation, the pyranose structure seems the more probable (91). Confirmatory evidence pointing to the same conclusion was obtained by Lucas & Stewart (92) in a study of the oxidation of alginic acid and methyl alginate with periodic acid followed by bromine. Each mannuronic acid unit with this reagent would be expected to yield a 2,3-dialdehyde which would be converted by bromine to a tricarboxylic acid. The former on hydrolysis would yield glyoxal and d-erythuronic acid, whereas the latter would give glyoxylic and *meso*tartaric acids. From the former, 42 per cent of glyoxal was obtained experimentally, and from the latter, 25 per cent of *meso*tartaric acid, indicating that carbon atoms 2 and 3 are not involved either in ring or bridge linkage.

Gums and mucilages.—The plant gums which ordinarily occur as exudations on the leaves or bark are salts of acid polysaccharides, the carboxyl groups of which are uronic in nature. The apparent homogeneity of the linkages in hemicelluloses and polyuronides, such as alginic acid, is in sharp contrast with the gums, from many of which sugar units may be removed by mild acid hydrolysis leaving behind a more resistant residue containing most, if not all, of the uronic acid groups. These acid groups may be readily titrated and accurate equiva-

lent weight figures obtained. Since the residues obtained by the progressive removal of sugar units usually have only one reducing group, a chain structure or some form of branched chain structure is probable.

Until recently studies on the gums have been primarily descriptive and limited to the determination of the various sugars and sugar acids involved. A beginning has now been made in the configurational study of the component units. It is to be emphasized that the plant gums from a particular botanical species are not necessarily homogeneous, and in fact wide differences are found in specific rotation and the proportions of the various constituents.

Gum arabic has been more extensively studied than other gums. Arabic acid preparations usually have an equivalent weight of from 1,000 to 1,200 and contain a nucleus, relatively resistant to hydrolysis, composed of galactose and glucuronic acids. To this nucleus are attached arabinose and methylpentose units which are readily removed by mild treatment with acid. Jackson & Smith have examined the degraded arabic acid obtained by hydrolysis with $0.01\ N\ H_2SO_4$, as a result of which l-arabinose, l-rhamnose, and a disaccharide, 3-d-galactosido-l-arabinose were removed. The resistant residue was apparently homogeneous and composed of galactose and glucuronic units in the proportions of three to one. By methylation and hydrolysis, 2,3,4,6-tetramethylgalactose, 2,3,4-trimethylgalactose, 2,4-dimethylgalactose, and 2,3,4-trimethylglucuronic acid were obtained in the relative molecular proportions of 1, 5, 3, and 3 respectively (93). From this it was concluded that the basic nucleus is composed of nine galactose residues and three glucuronic residues, all of pyranose form, linked by both 1,3- and 1,6-glycosidic linkages to give a branched chain structure probably having four terminal or "end" residues but only one reducing group. Three of the terminal residues must be composed of uronic groups, and one of galactose, since these were isolated in the form of completely methylated derivatives. A hexamethyl-6-β-glucuronosidogalactose was obtained by hydrolysis of the methylated arabic acid nucleus with cold $14\ N$ sulphuric acid, and the isolation of this aldobionic acid was taken to indicate that the side chains do not consist of a single unit of glucuronic acid but rather a terminal glucuronic group linked through at least one galactose residue with the main galactose chain (94). Additional information was provided by the isolation of the disaccharide 3-galactosidogalactose from

the products of autohydrolysis of degraded arabic acid (95). The basal structure therefore may be indicated as
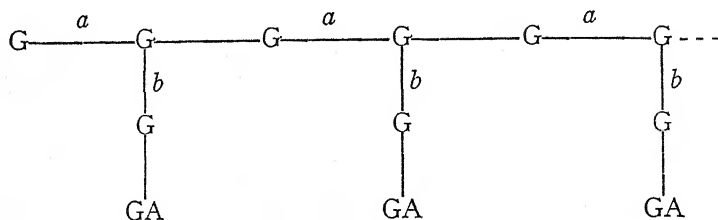
in which G = galactose and GA = galacturonic acid. The linkages *a* and *b* must be either of the 1,3 or the 1,6 type though it cannot yet be said in which position these linkages occur.

By direct methylation of arabic acid it has been established that the labile sugar residues are joined to the more resistant nucleus in the form of *l*-arabofuranose, *l*-rhamnopyranose, and 3-galactopyranosido-*l*-arabofuranose. The separation and identification of the various constituents of the mixture of methylated glycosides obtained from arabic acid is a difficult problem. In addition to the 1,3 and 1,6 linkages found in the degraded arabic acid, the presence of a 1,4 linkage has been detected by the isolation of 2,3-dimethylmethylglucuronoside. A further implication of the isolation of this derivative is that while in the degraded arabic acid all the uronic units constitute end groups, this is not the case with the original acid in which some of the groups must occupy intermediate positions in side chains. This might take place through an extension of the side chains indicated diagrammatically above by linkage of a sugar unit at position 4 of the glucuronic acid residue (96).

A somewhat similar study has been made of damson gum, from which *l*-arabinose may be removed by mild hydrolysis (97). The identification of the hydrolysis products from the fully methylated resistant nucleus indicated that this polysaccharide must also have a complicated branched structure. The derivatives included 2,3,4,6-tetramethyl-, 2,3,6- and 2,3,4-trimethyl-, and 2,3-dimethyl-*d*-galactose; 2,3,4-trimethyl- and 2,3-dimethyl-*d*-glucuronic acid; a small quantity of 2,3,4-trimethyl-*d*-xylose; and an unidentified derivative of *d*-mannose. The precise arrangement of the component sugars cannot be decided on this evidence alone (98).

Cherry gum has also been examined and found to contain 11.9

per cent urone and 57 per cent pentosan. The pentose present was almost exclusively *l*-arabinose though a small amount of *d*-xylose was detected. *d*-Galactose and *d*-mannose were present along with *d*-glucuronic acid. The probable proportions of these components were: arabinose, six moles; galactose, two moles; mannose, one mole; *d*-glucuronic acid, one mole. However, if the small amount of xylose present represents one unit in the molecule the molecular weight of the polysaccharide must be at least 7,500 (99). A preliminary study of gum ghatti would suggest that the general structure is not dissimilar to the other gums described, though the pentose content is distinctly lower than in many other cases (100).

The distinction usually drawn between gums, mucilages, and gel-forming substances like agar is quite indefinite, though it may be possible in the near future to group these various polysaccharides according to the general structure and components of the molecule. The mucilage from the bark of *Ulmus fulva* (slippery elm mucilage) was degraded by autohydrolysis followed by acid hydrolysis with *N* sulphuric acid for nine hours. As a result of this hydrolysis an aldobionic acid, identified as rhamnose-galacturonide, was isolated (101). The structure was determined as 2-*d*-galacturonido-*l*-rhamnose, the uronic acid having a pyranose ring. This aldobionic acid is apparently identical with that isolated from flaxseed mucilage (102).

The nature of agar has been established as a galactan with sugar units of unusual structure (103, 104). The alkali extract of *Gelidium amansii* contains a polysaccharide free of uronic groups, but from which *d*-galactose, *l*-arabinose, and a methyl-pentose, probably fucose, were isolated (105). Carrageen mucilage from *Chrondrus chrispus* is a polysaccharide ester of sulphuric acid, by the acetolysis of which two galactans were isolated (106).

Uronic acids.—The constitutional studies on various polyuronides have called for knowledge of the reactions and derivatives of uronic and aldobionic acids. Considerable progress has been made in this field. The developments several years ago following the isolation of immunologically important specific polysaccharides, which were found to contain uronic and aldobionic groups, did much to set this work in progress. Only three uronic acids, *d*-glucuronic, *d*-galacturonic, and *d*-mannuronic acid, are known to occur in natural compounds, but others can be obtained by synthesis (107). Keturonic acids have been obtained by the bromine oxidation of xylose, arabinose, and even glucosamine (108). Levene and co-workers have attempted the conver-

sion of uronic acids into the corresponding hexoses by reduction under pressure in the presence of Raney's catalyst. The aldobionic acid from gum arabic has been reduced to the corresponding disaccharide in two ways (109), and 2,3,4-trimethyl-*d*-galacturonic acid has been reduced to 3,4,5-trimethyl-*l*-galactonic acid (110). Reeves (111) has examined the structure of trimethylglucurone, and Link *et al.* (112) the derivatives of aldehydo-*d*-galacturonic acid.

The determination of uronic groups in polysaccharide or cellulosic materials of low uronic content is not wholly satisfactory since small quantities of carbon dioxide may be evolved from hexose or hexosan groups (113, 114). It has been shown that the rate of evolution of carbon dioxide may be used to indicate the presence of uronic groups in such materials (114). The precise conditions that may be employed under such circumstances have been investigated by Whistler *et al.* (115). Kapp (116) has devised a microprocedure for the determination of free uronic acids based on the Tollens reaction with naphthoresorcinol.. It remains to be ascertained yet whether the benzimidazole derivatives of the uronic acids can be made use of in their characterization as satisfactorily as for the seven aldomonosaccharides, the constants of which have been determined (117). The procedure depends on oxidation to the corresponding aldonic acid and coupling with *o*-phenylenediamine.

LITERATURE CITED

1. HAWORTH, W. N., *Chemistry & Industry,* 917 (1939)
2. HUSEMANN, E., *Papier-Fabr.,* **36,** 559 (1939)
3. HESS, K., AND GRIGORESCU, D., *Ber.,* **73B,** 499 (1940)
4. HESS, K., GRIGORESCU, D., STEURER, E., AND FRAHM, H., *Ber.,* **73B,** 505 (1940)
5. FREUDENBERG, K., AND BOPPEL, H., *Naturwissenschaften,* **28,** 264 (1940)
6. ARCHBOLD, H. K., *New Phytologist,* **39,** 185 (1940)
7. NORMAN, A. G., *J. Am. Soc. Agron.,* **31,** 751 (1939)
8. MOROSOV, A. S., *Compt. rend. acad. sci. U.R.S.S.,* **24,** 407 (1939)
9. ARCHBOLD, H. K., *Ann. Botany,* n.s. **2,** 183, 403 (1938)
10. HAWORTH, W. N., HIRST, E. L., AND LYNE, R. R., *Biochem. J.,* **31,** 786 (1937)
11. KIHARA, Y., *J. Agr. Chem. Soc. Japan,* **15,** 348 (1939)
12. SCHLUBACH, H., AND LOOP, W., *Ann.,* **523,** 130 (1936); SCHLUBACH, H., AND PEITZNER, H., *Ann.,* **530,** 120 (1937); SCHLUBACH, H., AND LENDZIAN, H., *Ann.,* **532,** 200 (1937); SCHLUBACH, H., AND BOË, H., *Ann.,* **532,** 191 (1937)
13. SCHLUBACH, H., AND BANDMANN, C., *Ann.,* **540,** 285 (1939)
14. VEIBEL, S., *Biochem. J.,* **32,** 1949 (1938)
15. LYNE, R. R., PEAT, S., AND STACEY, M., *J. Chem. Soc.,* 237 (1940)
16. HASSID, W. Z., *J. Am. Chem. Soc.,* **61,** 1223 (1939)
17. PEAT, S., SCHLÜCHTERER, E., AND STACEY, M., *J. Chem. Soc.,* 581 (1939)
18. HASSID, W. Z., AND BARKER, H. A., *J. Biol. Chem.,* **134,** 163 (1940)
19. DAKER, W. D., AND STACEY, M., *J. Chem. Soc.,* 585 (1939)
20. PETERSON, F. C., BARRY, A. J., UNKAUF, H., AND WISE, L. E., *J. Am. Chem. Soc.,* **62,** 2361 (1940)
21. OWENS, H. S., *J. Am. Chem. Soc.,* **62,** 930 (1940)
22. HUSEMANN, E., *Naturwissenschaften,* **27,** 595 (1939)
23. SCHMIDT, E., TANG, W. T., AND JANDEBEUR, W., *Cellulosechem.,* **12,** 201 (1931)
24. RITTER, G. J., AND KURTH, E. F., *Ind. Eng. Chem., Ind. Ed.,* **25,** 1250 (1933)
25. VAN BECKUM, W. G., AND RITTER, G. J., *Paper Trade J.,* **104,** No. 19, 49 (1937)
26. ANONYMOUS, *Paper Trade J.,* **110,** No. 22, 27 (1940)
27. JAYME, G., STORCH, K., KERLER, E., AND SCHWAB, G., *Papier-Fabr.,* **37,** Tech.-Wiss. Tl., 57 (1939)
28. STORCH, K., AND MÜLLER, O., *Papier-Fabr.,* **37,** Tech.-Wiss. Tl., 213 (1939)
29. JAYME, G., AND KERLER, E., *Holz Roh- u. Werkstoff,* **3,** 2 (1940)
30. COHEN, W. E., AND DADSWELL, H. E., *J. Council Sci. Ind. Research,* **12,** 115 (1939)
31. DADSWELL, H. E., AND ELLIS, D. J., *J. Council Sci. Ind. Research,* **13,** 129 (1940)
32. MACKNEY, A. W., *J. Council Sci. Ind. Research,* **13,** 115 (1940)

33. Van Beckum, W. G., and Ritter, G. J., *Paper Trade J.,* **108**, No. 7, 27 (1939)
34. Van Beckum, W. G., and Ritter, G. J., *Paper Trade J.,* **109**, No. 22, 107 (1939)
35. Wise, L. E., Peterson, F. C., and Harlow, W. M., *Ind. Eng. Chem., Anal. Ed.,* **11**, 18 (1939)
36. Reid, J. D., Nelson, G. H., and Aronovsky, S. I., *Ind. Eng. Chem., Anal. Ed.,* **12**, 255 (1940)
37. Ritter, G. J., and Mitchell, R. L., *Paper Trade J.,* **108**, No. 6, 35 (1939)
38. Preston, R. D., and Allsopp, A., *Biodynamica,* No. 53 (1939)
39. Gross, S. T., Clark, G. L., and Ritter, G. J., *Paper Trade J.,* **109**, No. 23, 37 (1939)
40. Preston, R. D., *Biol. Rev. Cambridge Phil. Soc.,* **14**, 281 (1939)
41. Sisson, W. A., *Chem. Rev.,* **26**, 187 (1940)
42. Farr, W. K., *Nature,* **146**, 153 (1940)
43. Hess, K., *Textil-Z. (Klepzig's),* **42**, 629 (1939)
44. Berkley, E. E., *Textile Research,* **9**, 355 (1939)
45. Hess, K., Kiessig, H., Wergin, W., and Engel, W., *Ber.,* **72B**, 642 (1939)
46. Mark, H., *J. Phys. Chem.,* **44**, 764 (1940)
47. Harlow, W. M., *Paper Trade J.,* **109**, No. 18, 38 (1939)
48. Harlow, W. M., *Paper Ind. Paper World,* **22**, 150 (1940)
49. Preece, I. A., *Biochem. J.,* **34**, 251 (1940)
50. O'Dwyer, M. H., *Biochem. J.,* **33**, 713 (1939)
51. O'Dwyer, M. H., *Biochem. J.,* **34**, 149 (1940)
52. Anderson, E., Seeley, M., Stewart, W. T., Redd, J. C., and Westerbeke, D., *J. Biol. Chem.,* **135**, 189 (1940)
53. Anderson, E., Hechtman, J., Seeley, M., *J. Biol. Chem.,* **126**, 175 (1938)
54. Krznarish, P. W., *Cereal Chem.,* **17**, 457 (1940)
55. Weihe, H. D., and Phillips, M., *J. Agr. Research,* **60**, 781 (1940)
56. Phillips, M., and Davis, B. L., *J. Agr. Research,* **60**, 775 (1940)
57. Norman, A. G., *The Biochemistry of Cellulose, the Polyuronides, Lignin, etc.* (Oxford, 1937)
58. Mitchell, R. L., and Ritter, G. J., *J. Am. Chem. Soc.,* **62**, 1958 (1940)
59. Voss, W., and Butter, G., *Ann.,* **534**, 161, 185 (1938)
60. Ploetz, T., *Ber.,* **72B**, 1885 (1939)
61. Ploetz, T., *Ber.,* **73B**, 57, 61, 74 (1940)
62. Khinchin, Y. G., *Bumazhnaya Prom.,* **17**, No. 12, 4 (1939)
63. Wise, L. E., *Paper Ind. Paper World,* **21**, 653 (1939)
64. Myers, P. B., and Baker, G. L., *Univ. Delaware Agr. Expt. Sta. Bull.,* No. 187 (1934)
65. Morell, S., Baur, L., and Link, K. P., *J. Biol. Chem.,* **105**, 1 (1934)
66. Henglein, F. A., and Schneider, G., *Ber.,* **69B**, 309 (1936)
67. Schneider, G., and Ziervogel, M., *Ber.,* **69B**, 2530 (1936)
68. Schneider, G., and Fritschi, U., *Ber.,* **69B**, 2537 (1936)
69. Hirst, E. L., and Jones, J. K. N., *J. Chem. Soc.,* 452 (1939)
70. Hirst, E. L., and Jones, J. K. N., *J. Chem. Soc.,* 454 (1939)

71. BEAVEN, G. H., HIRST, E. L., AND JONES, J. K. N., *J. Chem. Soc.,* 1865 (1939)
72. SMITH, F., *Chemistry & Industry,* 363 (1939)
73. LUCKETT, S., AND SMITH, F., *J. Chem. Soc.,* 1106 (1940)
74. LUCKETT, S., AND SMITH, F., *J. Chem. Soc.,* 1114 (1940)
75. BEAVEN, G. H., AND JONES, J. K. N., *Chemistry & Industry,* 363 (1939)
76. LEVENE, P. A., MEYER, G. M., AND KUNA, M., *Science,* **89,** 370 (1939)
77. KERTESZ, Z. I., *J. Am. Chem. Soc.,* **61,** 2544 (1939)
78. MOTTERN, H. H., AND COLE, H. L., *J. Am. Chem. Soc.,* **61,** 2701 (1939)
79. MANVILLE, I. A., REITHEL, F. J., AND YAMADA, P. M., *J. Am. Chem. Soc.,* **61,** 2973 (1939)
80. MATIDA, S., AND TASIRO, T., *J. Agr. Chem. Soc. Japan,* **15,** 243 (1939)
81. BOCK, H., AND EINSELE, R., *J. prakt. Chem.,* **155,** 225 (1940)
82. HINTON, C. L., *Dept. Sci. Ind. Research (Brit.), Food Invest. Special Repts.,* No. 48 (1939)
83. BAKER, G. L., AND GOODWIN, M. W., *Univ. Delaware Agr. Expt. Sta. Bull.,* No. 216 (1939)
84. BENNISON, E. W., AND NORRIS, F. W., *Biochem. J.,* **33,** 1443 (1939)
85. HINTON, C. L., *Biochem. J.,* **34,** 1211 (1940)
86. NIEMANN, C., AND LINK, K. P., *J. Biol. Chem.,* **100,** 407 (1933)
87. MIYAKE, S., AND HAYASI, K., *J. Soc. Trop. Agr. Taihoku Imp. Univ.,* **11,** 95 (1939)
88. MIYAKE, S., AND HAYASI, K., *J. Soc. Trop. Agr. Taihoku Imp. Univ.,* **11,** 204 (1939)
89. LUCAS, H. J., AND STEWART, W. T., *J. Am. Chem. Soc.,* **62,** 1070 (1940)
90. HIRST, E. L., JONES, J. K. N., AND JONES, W. O., *J. Chem. Soc.,* 1880 (1939)
91. HIRST, E. L., JONES, J. K. N., AND JONES, W. O., *Nature,* **143,** 857 (1939)
92. LUCAS, H. J., AND STEWART, W. T., *J. Am. Chem. Soc.,* **62,** 1792 (1940)
93. SMITH, F., *J. Chem. Soc.,* 1724 (1939)
94. JACKSON, J., AND SMITH, F., *J. Chem. Soc.,* 74 (1940)
95. JACKSON, J., AND SMITH, F., *J. Chem. Soc.,* 79 (1940)
96. SMITH, F., *J. Chem. Soc.,* 1935 (1940)
97. HIRST, E. L., AND JONES, J. K. N., *J. Chem. Soc.,* 1174 (1938)
98. HIRST, E. L., AND JONES, J. K. N., *J. Chem. Soc.,* 1482 (1939)
99. JONES, J. K. N., *J. Chem. Soc.,* 558 (1939)
100. HANNA, D., McREYNOLDS, L., AND SHAW, JR., E. H., *Proc. S. Dakota Acad. Sci.,* **19,** 130 (1939)
101. GILL, R. E., HIRST, E. L., AND JONES, J. K. N., *J. Chem. Soc.,* 1469 (1939)
102. TIPSON, R. S., CHRISTMAN, C. C., AND LEVENE, P. A., *J. Biol. Chem.,* **128,** 609 (1939)
103. PERCIVAL, E. G. V., *Pharm. J.,* **142,** 189 (1939)
104. FORBES, I., AND PERCIVAL, E. G. V., *J. Chem. Soc.,* 1844 (1939)
105. MIYAKE, S., AND HAYASI, K., *J. Soc. Trop. Agr. Taihoku Imp. Univ.,* **11,** 200 (1939)
106. DILLON, T., AND O'COLLA, P., *Nature,* **145,** 749 (1940)
107. STACEY, M., *J. Chem. Soc.,* 1529 (1939)

108. Crews, L. T., Hart, J. P., and Everett, M. R., *J. Am. Chem. Soc.,* **62,** 491 (1940)
109. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.,* **125,** 345 (1938)
110. Tipson, R. S., *J. Biol. Chem.,* **125,** 341 (1938)
111. Reeves, R. E., *J. Am. Chem. Soc.,* **62,** 1616 (1940)
112. Dimler, R. J., and Link, K. P., *J. Am. Chem. Soc.,* **62,** 1216 (1940)
113. Campbell, W. G., Hirst, E. L., and Young, G. T., *Nature,* **142,** 912 (1938)
114. Norman, A. G., *Nature,* **143,** 284 (1939)
115. Whistler, R. L., Martin, A. R., and Harris, M., *J. Research Natl. Bur. Standards,* **24,** 13 (1940)
116. Kapp, E. M., *J. Biol. Chem.,* **134,** 143 (1940)
117. Moore, S., and Link, K. P., *J. Biol. Chem.,* **133,** 293 (1940)
118. Ehrlich, F., summarized in *Abderhalden's Handb. biol. Arbeitsmeth., Abt. XI,* 1503 (1936)

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CHEMISTRY OF AMINO ACIDS AND PROTEINS[1]

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The present review contains only an incidental report on the chemistry of surfaces, the proteolytic enzymes, and the sulfur-containing amino acids and proteins, since these topics are treated elsewhere in this volume of the *Review*. Inasmuch as the two previous reviews have stressed the physical chemistry of the proteins and amino acids, the present writer has endeavored to emphasize more particularly the purely chemical aspect of the subject. The scope of the review is limited further, of necessity, because of the inaccessibility of current European journals. With a few exceptions, however, it has been possible to examine all of the papers cited in *Chemical Abstracts*.

Amino Acids

Stereochemical relationships.—It is now well established that the naturally occurring amino acids belong to the *l*-series of configurationally related compounds. It seems highly desirable, therefore, that the natural amino acids should be designated as $l(+)$ or $l(-)$ and the unnatural ones as $d(+)$ or $d(-)$, rather than by the older nomenclature. The sign of the optical rotatory power is denoted by the prefixes $(+)$ and $(-)$, although there has been no stipulation of the conditions under which the sign is to be determined. Inasmuch as the direction of rotation of $l(+)$-alanine (1) and $l(+)$-aspartic acid (2) in water changes sign with increase in temperature when other conditions are constant, a suitable temperature standard should be adopted. If it is assumed that the sign of the prefix is determined by the direction of the rotation of the free amino acid in water at 25°, the rule would be in accord with the usual designations of sign.

The chemical and optical bases on which the stereochemical relationships of the amino acids have been established are set forth in a number of review articles (3 to 8). The principles of these methods

and the amino acids to which they have been applied are given in the following summary:

Natural alanine has been shown to be configurationally related to $l(+)$-lactic acid. The amides or esters of the acetyl, benzoyl, and other N-derivatives of natural alanine and of derivatives of $l(+)$-lactic acid, but not of $d(-)$-lactic acid, show analogous changes in optical rotation (9, 10).

Amino acids or other compounds of the l-series and natural amino acids of unknown spatial relationship have been shown by chemical or optical methods, or by a combination of these, to be configurationally related. By a series of reactions in which there was no opportunity for a Walden inversion, natural serine was converted to $l(+)$-alanine and natural cystine (11, 12); natural asparagine to $l(-)$-serine (13); natural histidine methyl ester to $l(+)$-benzoylaspartic acid (14); the ethyl ester of N-benzoyl natural tyrosine to $l(+)$-aspartic acid (15); natural phenylalanine and $l(-)$-tyrosine to the same hexahydrophenylalanine (16); $d(+)$-crotylglycine to $d(-)$-benzoylaspartic acid and unnatural $d(-)$-norleucine (17); the corresponding derivatives of natural leucine and $l(+)$-aspartic acid to products having approximately the same optical rotation and essentially alike in chemical composition and structure (18); $l(+)$-aspartic acid and natural glutamic acid to α, ω-diamino acid derivatives which showed changes in optical rotation analogous to the corresponding derivatives of natural ornithine and lysine (19); $l(-)$-tyrosine and natural thyroxine to the same thyronine (20); and natural alanine and valine to products which, in accordance with the predictions, were optical antipodes (21).

Corresponding derivatives of natural alanine, asparagine, glutamic acid, isoleucine, leucine, phenylalanine, serine, tyrosine, and valine, and $(+)$-α-aminobutyric acid show analogous changes in optical rotation (1); solutions of natural asparagine, aspartic acid, and glutamic acid in water, acids, bases, and salts show analogous changes in optical rotation (1, 2); and acid solutions of natural alanine (22), arginine (23), aspartic acid (22), cystine (22), glutamic acid (22), histidine (23), hydroxyproline (23), hydroxyglutamic acid (24), diiodotyrosine (25), isoleucine (26), leucine (22), lysine (23), norleucine (27), proline (23), serine (28), tryptophane (23), tyrosine (22), and valine (29) show increasing positive rotations with increasing molal ratios of acid to amino acid.

Natural threonine is a member of the l-series of amino acids, since

it has the *l*-configuration of the groups on the α-carbon atom. It is designated as $d(-)$, however, because by reduction to $l(+)$-α-amino-butyric acid and oxidation to $d(-)$-lactic acid, its spatial configuration has been shown to be analogous to that of $d(-)$-threose (30).

It has been reported recently by Cadden (31) that natural canavanine belongs to the *l*-series of the amino acids since its rotation becomes more positive with increasing molecular equivalents of hydrochloric acid.

Synthesis.—Checked procedures for the synthesis of five amino acids have appeared in *Organic Syntheses*, 19 (1939) and 20 (1940). The amino acids which have been prepared are lysine mono- and dihydrochlorides, by the Beckmann rearrangement of cyclopentanone oxime (32); β-phenylalanine, by the catalytic reduction and hydrolysis of acetaminocinnamic acid (33), a method which appears to be less convenient than the azlactone procedure described in an earlier volume (34); serine, by a series of reactions from methyl acrylate and mercuric acetate (35); threonine, by a series of reactions from crotonic acid and mercuric acetate (36), in which threonine is separated from allothreonine by the fractional crystallization of the formyl-O-methyl epimers; and valine, by the amination of α-bromoisovaleric acid (37).

A simplified procedure for the preparation of ethyl-α-bromo-β-methoxypropionate, intermediate in the synthesis of serine by the method of Carter & West (35), has been described by Wood & du Vigneaud (38). The ethyl-α,β-dibromopropionate, prepared by the reaction of bromine and ethyl acrylate, was converted by sodium ethylate to ethyl-α-bromo-β-ethoxypropionate. This product was hydrolyzed and the serine isolated in 47 per cent of the over-all yield. Mayeda *et al.* (39) have proposed modifications in the experimental procedure recommended by Mitra (40) for the synthesis of serine from monochlorodimethyl ether and sodium phthalimidomalonic ester. The synthesis (41), resolution (42), and conversion to threonine (43) of allothreonine have been described by Carter and co-workers. The synthesis of threonine from acetoacetic ester, investigated without success by McIlwain & Richardson (44), was completed by Adkins & Reeve (45). Acetoacetic ester was nitrosated to oximinoacetoacetic ester and the latter ethylated and hydrogenated to threonine and allothreonine, which were separated by fractional crystallization from a mixture of water and ethanol.

The condensation of the appropriate alkyl halides with the sodium

derivative of ethyl benzamidomalonate, suggested by Redemann & Dunn (46) as a general method for the synthesis of α-amino acids and utilized for the preparation of seven amino acids, has been applied by Painter (47) to the synthesis of norleucine, α-amino-γ-phenoxy-*n*-butyric acid, and the lactone of α-amino-γ-hydroxy-*n*-butyric acid. The author's attempted synthesis of β- and γ-halogen amino acids by this method failed. A simple and inexpensive procedure for the preparation of the intermediate substance, benzoylaminomalonic ester was described.

The need in studies on bacterial nutrition for amino acids free from biologically important impurities, and the lack of serviceable methods for the synthesis of some of the more complex amino acids were emphasized by McIlwain & Richardson (44). These authors extended the catalytic reduction of oximino esters, which was applied by Harington & Randall (48) to β-hydroxyglutamic acid, to glutamic acid and hydroxyprolines (*a*) and (*b*). The synthesis of the hydroxyprolines by the same procedure was reported (49) in the same year (1938). The most satisfactory synthesis of glutamic acid is based on the Michael condensation of methyl acrylate and ethyl phthalimidomalonate, described by Marvel & Stoddard (50). The racemization of *l*(+)-glutamic acid with heat (51) is a convenient procedure for the preparation of large amounts of anhydrous or monohydrated *dl*-glutamic acid.

The Schmidt hydrazoic acid reaction, in which the distal carboxyl group in α-aminodicarboxylic acids is replaced by an amino group, has been applied by Adamson (52) to the synthesis of small quantities of (+)-α,γ-diamino-*n*-butyric acid, *dl*-ornithine, and *dl*-lysine. The general method for the preparation of α-amino-β-hydroxy acids by addition reactions of mercuric acetate to acrylic acid derivatives, first used by Abderhalden & Heyns (53) in the preparation of α-amino-β-hydroxy-*n*-butyric acid (threonine), has been adapted by Abderhalden (54) to the synthesis of β-hydroxyleucine and β-hydroxynorleucine. The Curtius degradation, adapted by Darapsky (55) to substituted cyanoacetic esters in the preparation of valine, leucine, and α-aminoisoamylacetic acid, has been applied by Gaudry & King (56) to the synthesis of β-phenylalanine.

A number of studies have been made of the autocondensation and ring-closure reactions of amino acid esters and other derivatives. Several new derivatives of β-phenylalanine-N-acetic acid and hydantoin-N-1-benzylacetic acid were described by Hahn *et al.* (57) while

Aspinall (58) has developed a practical synthesis for the hitherto unknown 2-ketopiperazine and its 3-alkyl and 3,3-dialkyl derivatives. The preparation and properties of diketopiperazines and polypeptide esters of amino acids are of particular interest in view of the classical studies of Curtius (59) and Meyer & Go (60) in this field. Frankel *et al.* (61) found that the tetraglycine ethyl ester, but not higher homologues, was formed when glycine ethyl ester stood for several weeks. Condensation of the latter to the ethyl esters of dodeca-, trideca-, and heptadecaglycine was observed, however, when the reactions occurred in xylene and benzene at room temperature and at the boiling point of these solvents. Hexadecaglycine ester was isolated from the mixture resulting from the reaction of oxygen and glycine ethyl ester. The ethyl esters of alanine and glycine carbamate also formed tetra- and other peptide esters which gave strong biuret reactions. Condensation products isolated by Pacsu (62) in a similar investigation were diketopiperazines from the methyl esters of glycine and glycylglycine, and hexapeptide ester from diglycylglycine methyl ester. Tetra- and pentapeptide esters did not react. When glycine hexapeptide ester was heated at 130° for 144 hours, or at 102° for a longer period, a polypeptide ester was isolated which had 0.56 per cent O-methyl content. These results indicate the absence of cyclization, and the formation of a polypeptide containing 96 glycine residues. Since this number is one third of the 288 amino acid residues postulated by Bergmann & Niemann (4) for proteins which possess a particle size of approximately 35,000 as determined by the ultra centrifugal method of Svedberg, a possible relationship is suggested between the described condensation reactions and protein synthesis *in vivo*.

The discovery by Jackson & Cahill (63) and Cahill & Burton (64), that optically active amino acids may be selectively racemized as well as acetylated (65) by ketene, is significant. When solutions of $l(-)$-leucine, $l(+)$-glutamic acid, and $l(-)$-tryptophane are kept continuously alkaline during the introduction of a stream of ketene gas no racemization occurs. On the other hand, rapid racemization results if ketene is allowed to generate acetic acid in excess of the alkali present. It is assumed that other amino acids will be affected similarly. In general, acetic anhydride and ketene are similar in their action on amino acids. According to the theory postulated by du Vigneaud and co-workers (66) it is surprising that abrine [N-methyl-$l(-)$-tryptophane] is racemized by ketene, since the asymmetric carbon

atom is not attached to a hydrogen atom capable of transient migration to oxygen in an intermediate lactone. When glycyl-$l(-)$-leucine and $l(-)$-leucylglycine were acetylated with ketene in alkaline solution the optically active derivative of each dipeptide was obtained; however, in acid solution, the acetyl derivative of glycyl-$l(-)$-leucine was completely inactive but that of $l(-)$-leucylglycine was fully active. Possible mechanisms by which these differences might be explained were discussed by Cahill & Burton. It was pointed out that ketene, because of its selective racemizing action, may be a convenient tool for the identification of terminal amino acids in peptides.

Ludwig & von Mutzenbecher (67) and von Mutzenbecher (68) have reported that thyroxine was formed when slightly alkaline solutions of iodine and casein (or silk fibroin, edestin, serum albumin, or serum globulin), or of sodium hydroxide and $l(-)$-diiodotyrosine were allowed to stand at 37° for two weeks. It appears that the first synthesis has been confirmed by Harington & Rivers (69) and the second by Block (70). Since the yield of thyroxine was low (20 mg. from 20 gm. of diiodotyrosine) it was possible that an impurity gave the result indicated. This possibility was obviated, however, by Block who employed dl-diiodotyrosine (synthetic) in his experiments. The theory that thyroxine is formed in these reactions, *in vitro* as well as *in vivo,* by the coupling of two molecules of diiodotyrosine with the loss of one side chain was suggested by Harington & Barger (71) in 1927.

Extensive studies, particularly those of Schoenheimer and associates, have been made on the preparation, properties, analysis, and metabolism of amino acids and other types of biological substances containing deuterium, isotopic nitrogen (N^{15}), and radioactive sulfur (S^{35}). Approximately sixty papers, and a number of review articles (72 to 77), have appeared since 1935, the date of the first publication (78) in this field.

The preparation of a number of new compounds containing isotopic atoms has been described during 1940. Two aldehydes, C_6H_5CDO and p-$C_6H_5C_6H_4CDO$, with deuterium substituted for hydrogen in the functional group, were prepared by reduction of the corresponding acid chlorides with deuterium (79). dl-N^{15}-Phenylaminobutyric acid, containing 2 atom per cent N^{15} excess, was synthesized by the catalytic hydrogenation of cinnamoylformic acid in the presence of isotopic ammonia containing 2 atom per cent N^{15} excess. The $l(+)$- and $d(-)$-N^{15}-isomers, each containing nearly 2 atom per cent N^{15} ex-

cess, were obtained by resolving the formyl derivative of the dl-N^{15}-compound with brucine as well as the dl-N^{15}-carbobenzoxy derivative with d- and l-α-phenylethylamines, although only the latter procedure gave optically pure products (80). dl-Deuterioornithine monohydrochloride, containing 12.5 atom per cent of stably bound α-β-γ-δ-deuterium, was synthesized from α-pyridone. The latter was hydrogenated to deuterio-α-piperidone which was hydrolyzed and d-aminovaleric acid was isolated as its m-nitrobenzoyl derivative. Bromination, amination, and hydrolysis of the m-nitrobenzoyl compound were carried out according to classical procedures (81). $l(+)$-N^{15}-Arginine, containing 8.33 atom per cent N^{15} excess and with the N^{15} in the amidine group, has been prepared by the degradation of α-toluenesulfoarginine with baryta to α-toluenesulfoornithine, treatment of the latter with isotopic methyl isourea (prepared from isotopic cyanamide), and hydrolysis of the resulting toluenesulfoarginine (82). Sarcosine, hydantoic acid, and guanidoacetic acid have been prepared from isotopic glycine, and methylhydantoic acid from isotopic sarcosine (83). Methionine (84), containing deuterium in the methyl group, has been synthesized by the reduction of homocystine with sodium in liquid ammonia and methylation with trideuteriomethyl iodide according to the procedure of du Vigneaud et al. (85). dl-N^{15}-Deuterioleucine, containing 6.49 atom per cent of N^{15} excess and 3.87 per cent deuterium bound to carbon, was synthesized from ethyldeuterio-α-bromoisocaproate and isotopic potassium phthalimide (86). Resolution (87) of the racemic amino acid, carried out with the brucine salt of the formyl derivative, gave $d(+)$-leucine containing 6.32 atom per cent N^{15} excess and 3.48 atom per cent deuterium and $l(-)$-leucine containing 6.54 atom per cent N^{15} excess and 3.60 atom per cent deuterium (88). Deuterio-dl-phenylalanine, containing 16.8 atom per cent deuterium or an average of 4.8 atoms of deuterium per molecule of phenylalanine, was prepared by maintaining a mixture of dl-phenylalanine and 84 per cent deuteriosulfuric acid, containing 54.5 atom per cent deuterium, at 50° for eight and a half days. It was demonstrated that the deuterium introduced was stably bound, that almost all of the deuterium was located in the phenyl ring, that the deuterium entered all five ring positions (probably to an equal extent), and that all hydrogen atoms in phenylalanine except those of the carboxyl and amino groups were stably bound (89).

In a similar study (90, 91) on the stability of hydrogen-carbon linkages in glutamic acid, it was shown that the hydrogen atoms of

the two carboxyl groups and of the amino group are labile (exchange at a very rapid rate), those in the α- and β-positions are stable (deuterium atoms not removed on prolonged boiling with 20 per cent hydrochloric acid), and those in the γ-position are semilabile (exchange half completed in about four days at 100° with 20 per cent hydrochloric acid in heavy water). Glutamic acid of the composition $C_5H_{8.968}D_{0.037}NO_4$ was formed when α-ketoglutaric acid was reduced with ordinary hydrogen and palladium in an ammoniacal solution of 6.7 atom per cent heavy water, and of the composition $C_5H_{7.61}D_{1.39}NO_4$, when the reduction was carried out with normal water and deuterium gas. The α-hydrogen atoms in the latter compound contained 26 atom per cent deuterium, and the β-hydrogen atoms 56 atom per cent. It appears, therefore, that β-hydrogen atoms of the α-ketoglutaric acid exchanged with the deuterium of the gas phase.

Additional syntheses to which only brief reference may be made because of the limitations of space are cysteic acid monohydrate, prepared by the oxidation of cystine with bromine (92); citrulline, by the acid hydrolysis of arginine (93); taurine, by the reaction of sodium sulfite with β-bromoethylamine hydrobromide (94); dl-homoarginine made from dl-lysine through the dicarbobenzoxy, ε-carbobenzoxy-α-benzoyl, and ε-guanidino-α-benzoyl derivatives (95); dl-6-methoxytryptophane, by the reduction with ammonium sulfide of 5(6′-methoxyindolylmethyl)-hydantoin prepared from o-nitro-p-toluidine (96); isocysteine (and isocystine), by the treatment with sodium sulfide of α-bromo-β-aminopropionic acid, which in turn was prepared from β-alanine and isoserine by the amination of β-chlorolactic acid (97); 3,5-diiodotyrosine, by treatment with iodine, and 3,5-dibromotyrosine by treatment with bromine, of anhydrohydroxydimercurityrosine (prepared from tyrosine and mercuric acetate) (98); and β-alanine methyl ester, from β-alanine and diazomethane (99).

Resolution.—The resolution of dl-cystine by means of the brucine salt of the acetyl derivative or the strychnine salt of the formyl derivative is unsatisfactory because the pure dl-form is obtained with difficulty by fractionation of the mixture of dl- and meso-cystines formed in the racemization of l(−)-cystine. Du Vigneaud et al. (100) circumvented this difficulty by reducing the mixture to S-benzyl-l(+)-cysteine followed by racemization of the latter with acetic anhydride in acid solution, resolution of the brucine salt of N-formyl-S-benzyl-dl-cysteine, and reduction of the S-benzyl-d(−)-cysteine to

$d(-)$-cystine. A nearly analogous procedure was employed in the resolution of inactive homocystine (101).

Resolution of amino acids by asymmetric enzymatic synthesis has been applied by Bergmann and co-workers to glutamic acid (102) and phenylalanine (103). The different speeds of enzymatic synthesis by cysteine-papain of the l- and d-anilides of carbobenzoxy-dl-glutamic acid and of acetyl-dl-phenylalanylglycine were utilized in the preparation in good yields of pure $d(-)$-glutamic acid, $l(-)$-phenylalanine, and $d(+)$-phenylalanine. Since the phenylalanine residue determines the course of the asymmetric synthesis but is not directly involved in the coupling reaction, it was shown that the natural asymmetric amino acid, $l(-)$-leucine, but not $l(-)$-proline, could replace the glycine residue in the enzymatic synthesis of the anilide of the acylated dipeptide.

Pacsu & Mullen (104) resolved dl-alanine by crystallizing, first, the strychnine salt of the less soluble benzoyl-$l(+)$-alanine and, second, the brucine salt of benzoyl-$d(-)$-alanine. That this procedure is more satisfactory than the use of these alkaloids in the reverse order, as recommended by Fischer (105), was observed in 1912 by Pope & Gibson (106). The method described by the latter authors has been shown to give satisfactory results in the reviewer's laboratory. Pacsu & Mullen give $[\alpha]_D^{20} = +10.33°$ for $l(+)$-alanine and $-10.30°$ for $d(-)$-alanine in 1 N HCl. Under essentially the same conditions, the following values have been given by other authors: $+14.7°$ and $+14.3°$ (1); $+13.2°$ and $+13.5°$ (107); and $+13.4°$ ($+9.55°$, value for the hydrochloride) for $l(+)$-alanine, and $-13.6°$ ($-9.68°$, value for the hydrochloride) for $d(-)$-alanine (105).

The resolution of dl-N[15]-deuterioleucine by classical methods has been carried out by Schoenheimer *et al.* (87, 88).

Chemical reactions and derivatives.—The β-anthryl-, 1,2-benzanthryl-3-, and 1,2-benzanthryl-1-isocyanates have been coupled with glycine and ε-aminocaproic acid in aqueous dioxane to give substances which are being tested for carcinogenic activity. The primary purpose of this and previous studies is to produce artificial protein antigens for use in immunization against the action of carcinogenic agents (108). The preparation and properties of mono- and di-α-furfurylglycines (109); of sulfhydryl and cysteine derivatives of carcinogenically active and inactive hydrocarbons (110); of the betaine hydrochlorides of dl-serine, dl-threonine, and dl-allothreonine (111); of polyoxyacyl derivatives of β-alanine analogous to pantothenic acid

(112); of 2,5-, 3,4-, and 3,5-diiodo-, and 2,3,5- and 3,4,5-triiodohippuric acids (113); and of the half picrate and monoflavianate of desaminocanavanine (114), have been described.

Although valine, phenylalanine, and leucine, when suspended in pyridine at room temperature, react readily with acyl chlorides to give good yields of acylated amino acids, the use of pyridine as an acylating medium was found to be unsatisfactory because of the insolubility of certain amino acids and complicating side reactions (115). It has been shown that only methionine, of the common natural amino acids, is convertible to crystalline methyl sulfonium salts of the composition $-OOC \cdot CH(NH_3^+)CH_2CH_2S(CH_3)R^+X^-$, by the action of methyl bromide or other alkyl halide in an acetic-formic acid medium. This reaction may provide a basis for the bioconversion of methionine to cysteine, the isolation of natural methionine, and the determination of methionine (116). The studies by Levene et al. (117, 118) on the high pressure catalytic reduction of esters of α-amino acids have been continued. Alcohols, aminohydrocarbons, piperazines, or diketopiperazines were derived from dl-leucine, dl-phenylalanine, and phenylaminoacetic acid esters by reduction over the Raney catalyst depending upon the temperature, duration of the reaction, and proportion of the catalyst. The object of this investigation is to obtain optically active alcamines for use in studying the configuration of the α-amino acids and the relationship of optical activity to physiological action.

Analysis.—Several new types of micro-Kjeldahl apparatus have been described during the preceding year (119, 120, 121, 122). The method of Keys (122) appears to be simple, rapid, and highly accurate, although a special vacuum still, microburette, measuring pipette, and micro-Kjeldahl apparatus are required.

Many different types of procedures have been devised for the determination of amino acids in blood, urine, and protein hydrolyzates although simple methods by means of which highly reproducible and accurate results may be obtained are largely lacking. Noteworthy progress has been made during the past few years.

A colorimetric test for methionine with sodium nitroprusside (123); the colorimetric analysis of histidine in urine by modified Knoop procedures (124, 125); and the photometric determination of tryptophane, tyrosine, diiodotyrosine, and thyroxine in protein hydrolyzates by the Millon reaction (126) have been described. It appears that tyrosine and tryptophane in a protein hydrolyzate can

be determined photometrically with an error of 2 per cent or less. Values for tryptophane in casein and other substances have been shown to be reliable when determined by the glyoxylic acid or ultraviolet spectrophotometric method but not by the *p*-dimethylaminobenzaldehyde procedure (127). Alanine and threonine in protein hydrolyzates have been determined quantitatively by oxidation of these amino acids, under special conditions, to acetaldehyde and the colorimetric analysis of the red color resulting from the condensation of acetaldehyde with *p*-hydroxydiphenyl (128).

The quantitative precipitation of an amino acid salt from protein hydrolyzates is seldom complete, because of the solubility of the salt in the mother liquor. For this reason Bergmann *et al.* (129, 130) have proposed the use of reagents which give salts possessing measurable solubility. Assuming that the amount of an amino salt which is precipitated at equilibrium is a function of the concentration of its ions in solution, these authors made use of the solubility products of glycine trioxalatochromiate and proline rhodanilate in determining these amino acids in gelatin and collagen. The validity of this principle for the determination of amino acids in proteins with the precision required to establish the amino acid composition of proteins is unquestioned. It was determined that the $d(+)$-antipode of proline could not have been more than 1.5 per cent of the total protein (131) and that the numerical ratios of proline to glycine to all amino acid residues in gelatin and collagen are approximately $3:7:21$. Bergmann *et al.* (132) have reported solubility products for the salts of a number of aromatic sulfonic acids with various amino acids. Of the twenty-six sulfonic acids listed it was shown that twenty-five form sparingly soluble salts with phenylalanine, twenty-two with leucine, seventeen with histidine, fourteen with arginine, thirteen with tyrosine, and three with lysine. The use of dilituric (5-nitrobarbituric) acid as a reagent for amino acids has been suggested by Redemann & Niemann (133) who reported the solubilities of the diliturates of a series of amino acids. In the estimation of histidine in protein hydrolyzates by the gravimetric determination of histidine nitranilate, recoveries of histidine ranging from 99 to 103 per cent were reported by Block (134). Vickery (135) has shown that the determination of arginine in protein hydrolyzates by precipitating it in acid solution, first as the slightly soluble diflavianate, and then as the monoflavianate, gives highly reproducible and apparently accurate results.

A new procedure for the quantitative analysis of amino acids and other types of organic compounds in mixtures by isotope dilution has been described in a significant paper by Rittenberg & Foster (136). A weighed amount x of a deuterio derivative of an amino acid of known deuterium content (C_0) is added to a protein hydrolyzate, a pure sample of the amino acid is isolated, the deuterium content (C) of the latter is determined, and the amount y of isotopically normal amino acid originally present in the mixture is calculated from the equation, $y = \left(\dfrac{C_0}{C} - 1 \right) x$. From a consideration of the theoretical errors, it was shown that the probable error is about 1.5 per cent when C_0 is equal to or greater than 5 per cent, C_0/C is equal to 10, and the compounds isolated are pure. This method has been applied to the determination of glycine, glutamic acid, and aspartic acid in fibrin; leucine in hemoglobin (137) and in the whole rat (87), and glutamic acid in malignant tumors (138).

The determination of the basic amino acids in proteins by an electrolytic method has been described by Albanese (139). Following the separation of these amino acids from other hydrolytic products by electrical transport, arginine is determined as the monoflavianate, histidine as the mercuric chloride complex, and lysine by a micro-Kjeldahl analysis of the final fraction. The manipulative errors are reduced to a minimum in this procedure which was shown by analysis of mixtures of pure amino acids to have both a precision and accuracy of about 1 per cent for arginine and histidine and approximately 3 per cent for lysine. A comparison is given of the results obtained in the analysis of various proteins by the electrolytic and other methods. The purity of methionine was determined with an accuracy of ± 0.1 per cent, and the methionine content of tissue extracts with an accuracy of 0.5 per cent, by Toennies and co-workers (140). The principle of the method is the oxidation of methionine by hydrogen peroxide (in aqueous perchloric acid) to the sulfoxide level. Negligible quantities of hydrogen peroxide are consumed by other natural amino acids except tryptophane, cystine, and cysteine.

The observation of Kendrick & Hanke (141) that glycine and cystine yield theoretical amino nitrogen values by the Van Slyke manometric method when a deaminizing mixture containing potassium iodide is used, has been confirmed by these authors (142). This effect of potassium iodide on cystine, but not glycine, was noted also by Dunn & Porush (143). The first authors advanced the theory

that iodine preferentially oxidizes cystine sulfide and deaminized glycine while the second authors proposed that the iodide effect on cystine may be explained by the formation of a mercuric iodide complex. Both laboratories found, however, that the amino nitrogen of blood filtrates, measured by methods not using potassium iodide, is reduced to about 15 per cent of the usual values by the use of the potassium iodide reagent. This result is particularly significant if the view is correct that reducing substances, other than amino acids, are the principal cause of the observed decrease in blood amino nitrogen.

A convenient wet combustion method for the determination of 0.3 to 15 mg. of carbon in amino acids and other types of organic compounds has been described by Van Slyke & Folch (144). By combustion of the substance with a mixture of chromic, iodic, sulfuric, and phosphoric acids the theoretical yield of carbon dioxide is given in one to three minutes. The carbon dioxide is collected and measured in the Van Slyke-Neill manometric apparatus. Gornall & Hunter (145) have shown that uramino acids react slowly with the nitrous acid in the Van Slyke apparatus and that two atoms of nitrogen are liberated only from those amino acids (isoleucine, valine, and α-amino isobutyric acid) in which the carbon chain branches at the α- or β-position.

Isolations.—An improved procedure for the isolation of pure $l(-)$-phenylalanine from casein and zein hydrolyzates in quantities of 2 gm. has been reported by Baptist & Robson (146). The principles utilized are the salting out with sodium chloride of a phenylalanine-containing "leucine-methionine" fraction or butyl alcohol extraction of monoamino acid fraction, isolation of the insoluble copper salts formed from one of the foregoing fractions, removal of copper and isolation of $l(-)$-phenylalanine picrolonate, and isolation of $l(-)$-phenylalanine from its picrolonate. Although Bergmann's (147) method for the isolation of $l(-)$-proline from gelatin as the rhodanilate was found by Mayeda (148) to be satisfactory, pure $l(-)$-hydroxyproline could be obtained by means of its Reineckate only after removal of the contaminant, lysine. Since it seems probable from the evidence presented by Van Slyke *et al.* (149) that the hydroxylysine previously crystallized from gelatin is a straight-chain compound and has the hydroxyl group and its more basic (non-α) amino group on adjacent carbon atoms, this diamino acid is considered to be either α,ε-diamino-γ-hydroxy- or α,δ-diamino-ε-hydroxy-*n*-caproic acid. Damodaran & Ramachandran (150) have found

the following ratios for paranuclein (casein phosphopeptone) : amino acid : phosphorus atoms, 10 : 3, and glutamic acid : isoleucine : serine, 3 : 3 : 4, rather than hydroxyglutamic acid : hydroxy-α-aminobutyric acid : serine, 3 : 4 : 2, as previously reported. Additional amino acids, whose isolation from protein sources has been reported, are γ-aminobutyric acid from liver (151), the dioxyacyl derivatives of β-alanine and $l(—)$-leucine from tuna liver (152), and basic amino acids from snake (cobra) muscles (153) and pig pancreas (154).

In the personal communication (which has now been published) from Chibnall et al. (155), referred to in the review of Dodds &. Dickens (156) on the optical forms of amino acids in tumors, it has been shown that only small amounts of partially racemized glutamic acid, as well as aspartic acid, can be isolated both from normal and malignant tissue proteins and that the occurrence of $d(—)$-glutamic acid is not a characteristic of malignancy. In recent publications, Dittmar (157) and Ottawa (158) have described the isolation of $d(—)$-glutamic acid from necrotic tumor material essentially by the Kögl & Erxleben technique. The view of Johnson (159) that the small amount of $d(—)$-glutamic acid, which was shown to be present in malignant, as well as normal, tissue, may be explained by slight racemization during hydrolysis of the protein is shared by Arnow & Opsahl (160). The latter authors assume, however, that partly racemized glutamic acid residue is present in proteins before hydrolysis or that a high degree of racemization during hydrolysis is induced by some property peculiar to malignant tissue, if the results of Kögl and Erxleben are to be explained. From experiments on proteins, normal and malignant tissue, dl-glutamic acid, and d-phenylaminobutyric acid, Lipmann et al. (161) demonstrated, with the aid of Krebs' specific d-α-amino acid oxidase, that small (0.6 to 3.7) percentages of unnatural amino acid antipodes are present in protein and tissue substrates. Similarly, Graff et al. (162) concluded from the analysis of six specimens of malignant tissue by the isotope dilution method that $d(—)$-glutamic acid, if present at all, could not have been more than 1.0 per cent of the total glutamic acid. It would seem to be a valid conclusion from these studies that there is no significant difference between normal and malignant tissue in the capacity to racemize amino acids. The occurrence of the unnatural antipodes of glutamic acid in the capsular material of certain bacteria (163), of tyrosine and valine in the tryptic digest of casein (164), and of proline in ergot alkaloids (165, 166) has been reported.

A significant investigation on the partition of N-acetyl, N-benzoyl, N-acetyl-O-benzoyl, and N-acetyl-O-methyl derivatives of amino acids between chloroform and water, ether and water, and ethyl acetate and water systems of immiscible solvents has been reported by Synge (167). This study recalls the pioneer work of Dakin on the extraction of amino acids from protein hydrolyzates with *n*-butyl and other alcohols and the investigation of England & Cohn on the distribution coefficients of amino acids between *n*-butyl alcohol and water. Calvery (168) has reviewed this earlier work. It was demonstrated that P (concentration of solute in the aqueous phase / concentration of solute in the organic solvent phase) is not markedly affected by c (concentration of solute in the aqueous phase) although P showed a negative temperature coefficient and was lowered for any given value of c by raising the concentration of neutral salt in the aqueous phase. Values for P ranged from about 5 to > 1000 for a series of acetamino acids. Because of the large differences between the partition coefficients of a homologous series of N-acetylamino acids, it was found possible to effect the separation of N-acetylmethionine and N-acetylproline from a mixture of fourteen N-acetylamino acids, which simulated a gelatin hydrolyzate, by extraction with chloroform in the Neuberger continuous liquid-liquid extractor. A method for the isolation of N-acetyl-O-benzoyl derivatives of the hydroxyamino acids (hydroxyproline, serine, threonine, and tyrosine) from a protein hydrolyzate was described and applied in preliminary experiments to fibrin, gelatin, and wool. A procedure for the isolation from protein hydrolyzates of the N-acetyl-O-methyl derivatives of hydroxyamino acids was proposed. The isolation of $l(-)$-hydroxyproline (5 gm. per 100 gm. gelatin) from a gelatin hydrolyzate by means of its N-acetyl-O-benzoyl derivative is of interest in view of its simplicity and the shortcomings of existing methods.

Nicolet & Shinn (169) have shown that periodic acid readily splits hydroxyamino acids bearing the hydroxyl and amino groups on adjacent carbon atoms with the formation of one mol each of ammonia and aldehyde (or ketone) characteristic of the hydroxyamino acid. It was demonstrated that formaldehyde is formed from serine and acetaldehyde from threonine. Identification of formaldehyde as an oxidation product of the hydroxylysine isolated from gelatin enabled Van Slyke *et al.* (149) to establish the possible structures of this diamino acid. A method for the quantitative analysis

of threonine, based on the oxidation of this hydroxyamino acid to acetaldehyde with lead tetraacetate, has been devised by Block & Bolling (170). Since none of the common amino acids gives either ammonia or aldehyde, oxidation with periodic acid and determination of ammonia affords, as suggested by Van Slyke et al., a simple method for the differentiation of hydroxyamino acids from those which do not have the $-CH(NH_2) \cdot CH(OH)-$ group. It was reported in a recent paper by Martin & Synge (171) that acetaldehyde is the only aldehyde formed by the oxidation with periodic acid of the amino acids in wool, gelatin, and casein hydrolyzates but that some propionaldehyde was identified in similar experiments with gluten. These aldehydes were identified as their 2,4-dinitrophenylhydrazones. The results with gluten suggested the presence of β-hydroxyvaline, or β-hydroxynorvaline, in this protein. Very recently, Martin & Synge (171a) have found that acetaldehyde, but not propionaldehyde, is liberated by periodate from gluten hydrolyzates. It was stated that

The explanation of our earlier findings seems to be that acetaldehyde-2,4-dinitrophenylhydrazone is dimorphous and the metastable form gives an x-ray powder photograph closely similar to that of propionaldehyde-2,4-dinitrophenylhydrazone, with which it readily forms mixed crystals, and is probably isostructural.

Physical chemistry.—In further investigations on the stereoisomerism of isoserine (172) and of γ-amino-β-hydroxybutyric acid (173), Tomita & Seiki have determined the physical properties and the structures of the N-benzoyl derivatives of the four enantiostereomers of each of these amino acids. It was established by means of roentgenographic diagrams that the *l*-I and *d*-I, as well as the *l*-II and *d*-II, pairs are optical antipodes, whereas the I- and II-compounds have entirely different structures. It would appear, from the evidence presented by Bergmann & Lissitzin (174) that these four optically active isomers may be represented as the *l*-; *d*-; *l,l,d*-; and *d,d,l*- forms, the last two being molecular compounds of the *dl*- with the *l*- and the *dl*- with the *d*-form.

The Raman spectra of glycine in water solution at nine pH levels from 0 to 13, of gelatin hydrolyzate at pH 0, and of peptone hydrolyzate at the three pH levels, 0, 0.1, and 4.5, have been determined by Goubeau & Lüning (175). The moderately intense lines, 504 and especially 868 cm.$^{-1}$ [the latter corresponding to 871 cm.$^{-1}$ observed by Edsall (176) for the sensitive frequency of

$^+NH_3$—CH_2—COOH], considered to be characteristic of glycine in acid solution, were also noted in the acid solutions of the protein hydrolyzates. It is suggested that determination of the lines of other amino acids may lead to a method for the analysis of protein hydrolyzates. The Raman spectra reported by Edsall & Scheinberg (177) for CH_3ND_2, $CH_3ND_3^+$, and analogous compounds, served to identify several frequencies uncertain in the spectra of substances containing amino and ammonium groups.

The reaction of formaldehyde with amino acids has been investigated by Baur (178) and Smith *et al.* (179). Proceeding from the assumption that $CH_2:NCH_2COOH$ is the product of the reaction between glycine and formaldehyde, the first author determined the apparent acid dissociation constant of methyleneglycine and the equilibrium constant of the reaction. It was calculated that, under specified conditions, the methyleneglycine is 22 per cent dissociated as a complex and 53 per cent as an acid. Smith *et al.* prepared the formaldehyde derivatives of free base arginine, lysine, and histidine. The empirical formula weights of the formaldehyde derivatives, calculated from their nitrogen content, were not in agreement with the molecular weight of the postulated methylol, heterocyclic ring, or methylene imino structure. It was observed from their x-ray diffraction patterns that the free bases of the three amino acids and the aged histidine-formaldehyde product are highly crystalline whereas the lysine-arginine and arginine-formaldehyde derivatives are amorphous. It would appear from the evidence presented by Levy (180), Tomiyama (181), and Wadsworth & Pangborn (182) that complexes other than those postulated by Baur and by Smith *et al.*, may be formed by the reaction of formaldehyde and amino acids.

The effects of sodium chloride, magnesium chloride, and other salts on the dissociation of glycine, alanine, aspartic acid, arginine, and ornithine have been investigated by Batchelder & Schmidt (183). The effects of single salts on the ionization of these amino acids could be quantitatively determined only in those experiments in which cells without liquid-junction potentials were used to measure the electromotive force. The effects of salts on the ionization of amino acids were explained on the theory of interionic attraction. By means of the assumption that only electrostatic forces determine the effects of salts on the ionization of proteins and amino acids and the general equation, $AH_n^+ = AH_m^+ + H^+$, where A represents the isoelectric ampholyte and the value of n is greater than that of m

by unity, equations were derived from the expression for the thermo-
dynamic equilibrium constant from which it was deduced that (a)

the addition of salts to a protein solution will cause an increase in the pH of the
solution if the initial pH was less than that of the isoelectric point and a de-
crease in pH if the initial pH was greater than that of the isoelectric point,

and (b)

at all points on the dissociation curves (of amino acids), except where n has a
value of unity, the effects of salts in low concentration correspond qualitatively
to the effects of salts on the dissociation curves of proteins.

The osmotic coefficients and activity coefficients of a series of
amino acids have been calculated by Smith & Smith (184, 185)
from isopiestic vapor pressure measurements. It was shown that
glycine is a more nearly perfect solute in water at 25° than at 0°
although the change in activity with temperature is small; that
osmotic and activity coefficients usually increase with each methylene
group in a homologous series; that the activity of a branched chain
amino acid is higher than that of the corresponding normal chain
acid; that the osmotic coefficient decreases with increase in dipole
moment and with substitution of the strongly polar hydroxyl group
in the hydrocarbon chain; and that N-methyl amino acids have
higher osmotic coefficients than the analogous nonmethylated forms.

The heats of dilution of aqueous solutions of glycine at 25° have
been measured by Sturtevant (186) and Gucker *et al.* (187). Pertzoff
(188) has determined that the experimental value for the molecular
refraction of the ions of aspartic acid is in better agreement with that
calculated from the Bjerrum type of ionization, than from the
classical. A comprehensive study of the optical properties of the
A^+, $A^\pm$, $^-A^\pm$, and $A^=$ ions of aspartic and glutamic acids has been pub-
lished by Pertzoff (189). A special polarimeter and tubes from 2 to
40 dm. in length were employed. Space does not permit an extended
discussion of this study, although one interesting conclusion which
may be mentioned is that the rotation of the $A^\pm$ ion of glutamic acid
varies linearly with the concentration while the rotation of aspartic
acid is proportional to the square root of the concentration. Refer-
ence may be made to the recent review by Kauzmann, Walter &
Eyring (190) on the theories of optical rotatory power.

The dielectric absorption of a series of amino acids and peptides
in water and water-ethanol has been measured by Parts (191). Dif-
ferences between the calculated and actual molal volumes were con-

sidered to be explained by electrostriction caused by zwitter ions. The chemical characteristics of the two ring-N atoms in histidine have been explained by Hill & Branch (192) from a consideration of the four types of resonance forms. Changes in pH observed by Maslov (193) in potassium chloride and sodium chloride solutions of glycine irradiated for twenty minutes in an ultrashort wave field were considered to be caused by special properties of glycine-salt complexes.

Peptides

The solubility of glycine anhydride at 20° in water and in 0.25 to 3 molar solutions of sodium, potassium, and lithium salts of the halogens has been measured by Meyer & Klemm (194). The solubility decreased with increasing concentration of potassium fluoride, but, except with the iodides (which caused an initial decrease), the solubility increased with all other salts tested.

The isolation from *Eisenia bicyclis* of a new polypeptide, pyroglutamyl-glutamyl-alanine, has been reported by Ohira (195). The alkaline hydrolysis of the N-acetyl derivatives of glycylglycine, glycylalanine, alanylglycine, and alanylalanine has been followed by Konikov & Lerman (196) using an electrometric titration procedure. It was determined from the quantities of acetic acid, dipeptide, and amino acid liberated during hydrolysis for twelve days that the percentages of peptide bonds hydrolyzed were from two to six times those of the acetoamide type. The relative ease of hydrolysis of peptide bonds was assumed to be associated with the formation of relatively unstable enol or hydroxyimine tautomers. The activities of five peptides of glycine and alanine in aqueous solution at 25° were determined by Smith & Smith (197).

Proteins

Insulin.—It has been determined by Linderstrøm-Lang & Jacobsen (198) that lactoglobulin (molecular weight, 38,000) contains 328 ± 10, and insulin (assumed molecular weight, 35,700) 292 ± 10, peptide bonds. These values are in close agreement with the numbers, 324 ($2^2 \times 3^4$) and 288 ($2^5 \times 3^2$), required by the Bergmann-Niemann periodicity hypothesis. By means of electron density projections of the insulin structure using available x-ray data, Huggins (199) has obtained evidence in favor of, and opposed to, the cage structure postulated by Wrinch. Du Vigneaud *et al.* (200)

have concluded that methionine is absent from insulin since tests were practically negative for homocysteine thiolactone and methyl iodide in the hydriodic acid digests of crystalline insulin. The large-scale preparation of zinc-insulin crystals for general clinical use in the treatment of diabetes has been described by Romans *et al.* (201). The yield by this process is 800 to 900 units per pound of beef pancreas.

Isolation, composition, chemical reactions, and analysis.—Structural and oxidative changes occurring when wool is ground to a powder were investigated by Routh (202). The effect of hot alcohol on the digestibility of beef liver and kidney globulins has been studied by Harris & Mattill (203). Rutherford & Harris (204) have shown that the alleged A and B fractions of sericin are not definite chemical entities.

A procedure for the quantitative determination of serum proteins based on the spectrometric and visual colorimetric analysis of the biuret color has been described by Robinson & Hogden (205). The biuret colors observed by Kosolapov (206) in tests with egg white were violet with Cu^+ (as well as Cu^{++}) salts, yellow with Ni^{++}, and reddish violet with Co^{++}. The taste ratio (ratio of glutamic acid nitrogen to total amino nitrogen) of soybean and wheat protein hydrolyzates has been determined by Kihara (207). Comparison of the percentages of five amino acids in zein, determined by micro and macro methods, has been made by Laine (208). Variations in the cystine and cysteine content of human hair with age, sex, and degree of pigmentation have been reported by Clay *et al.* (209). The acid-binding capacities of egg albumin and of the proteins of wheat flour have been determined by titration of the precipitate formed from metaphosphoric acid and water-soluble proteins. This phenomenon is explained, according to Briggs (210), by the formation of a polyvalent metaphosphate ion-protein complex in which the amino groups are masked and the carboxyl groups become titratable because of the shift in their dissociation constant. Brand & Kassell (211) have found that porphyrindin is reduced by tyrosine, as well as cysteine, but not by other common amino acids. Analyses were made of the reducing groups of egg albumin liberated by heat denaturation and by the action of guanidine hydrochloride.

Conjugated horse serum albumin containing twelve and eighteen benzanthryl prosthetic groups per molecule has been prepared by Creech & Jones (212). Jung (213) has investigated the differences in

absorption spectra for the thiocyanogen, cyano, azid, and thio deriva-
tives of methemoglobin. The nitrogen content of proteins and re-
lated substances and the hydrogen evolved in reactions with sodium
in liquid ammonia have been correlated by Miller & Roberts (213a).
The initial rapid production of iodide in the reaction between iodo-
acetic acid and denatured egg albumin was shown by Rosner (214)
to be caused by sulfhydryl groups. A slow reaction of unidentified
groups was also observed. Determinations of the tyrosine and trypto-
phane content of the thoracic muscles of Leghorn chickens, a hawk,
golden eagle, grouse, pheasant, and cockatoo have been reported by
Gurevich (215). No significant differences were noted.

The homogeneity of equine encephalomyelitis virus protein
preparations has been determined by Taylor *et al.* (216) by means
of photometer curves from sedimentation diagrams. The isoelectric
points of tobacco, potato, and aucuba mosaic viruses, determined by
means of turbidity-pH measurements, have been shown by Pfan-
kuch (217) to be approximately 3.2, 3.2, and 3.7, respectively.
Mutant modifications of tobacco mosaic virus have been prepared
by Pfankuch *et al.* (218) by radiating the ordinary virus form with
12,000 to 14,000 Röntgen units or the γ-rays from 10 mg. of meso-
thorium. Stanley (219) has reviewed recent developments in the
field of the viruses.

Other investigations, to which only brief reference may be made, include the
effect of protein dissociating agents and x-rays on the structural viscosity of
protoplasm (220); fractionation and properties of horse serum proteins (221);
the bound carbohydrate of serum proteins (222); carbohydrates in hen ovitellin
and lobster and crabmeat proteins (223); the iron proteins of spleen (224);
proteins from the dried latex of *Hevea brasiliensis* (225); proteins from dog
muscles (226); absorption spectra of hen and quail ovitellin (227); fractionation
of soybean protein (228); absorption spectrum of vitellin from the yolk of log-
gerhead turtle eggs (229); absorption spectra of wheat glutenin (230); prepara-
tion of thrombin (231); preparation of phytothrombin free from papain (232);
preparation and metabolic properties of potato and green bean proteins (233);
action of formaldehyde on collagen in tanning (234); protein nature of sperm
agglutinins of the keyhole limpet and the sea urchin (235); absorption of amino
acids by erythrocytes (236); blood albumin-globin ratio (237, 238); composi-
tion of urinary and serum proteins (239); and a crystalline albumin component
of skeletal muscle (240).

Denaturation.—It has been shown by Edsall *et al.* (241, 242)
that the titratable sulfhydryl groups of rabbit myosin are reduced
to zero while the double refraction of flow and viscosity of myosin
solutions are diminished by the action of denaturing agents. Bull

(243) has measured the viscosities of native, heat-denatured, and urea-denatured egg albumin and calculated the asymmetry of these molecular forms. It was concluded that even urea-denatured egg albumin has considerable structure and is not simply a polypeptide chain in the β-keratin form. The rate of denaturation of methemoglobin by alkali was found by Conn *et al.* (244) to increase rapidly with pH. It was determined that, at constant pH, the heat of denaturation is about 100 kcal. Investigations by Roche & Chouaïech (245, 246) on native, denatured, and reversed albumin and hemoglobin led to the conclusion that denaturation is not completely reversible. The compressibility and amino acid composition of monolayers of native and denatured proteins have been correlated by Langmuir & Waugh (247). Other studies which have been reported are the reversibility of the denaturation of silk fibroin (248); interaction of denatured and native egg albumin with hydrogen and hydroxyl ions (249); immunological properties of acid-denatured egg albumin (250); denaturation of soybean albumins (251); a review of fibrin coagulation as a polymerization-crystallization process (252); review of direct and indirect methods, and description of an optical-kinetic method, for the determination of coagulation with a photocell sensitive to infrared light waves (253); and x-ray analysis of albumin denaturation (254).

Electrochemical properties.—The electrophoresis apparatus and methods of Tiselius have been described in recent reviews by Tiselius (255). In studies on the influence of electrolytes on the electrophoretic mobilities of proteins, Abramson (256) found that the mobilities of serum albumin and pseudoglobulin from pH 4 to 8 are proportional to the combining power of the proteins with hydrogen and hydroxyl ions. Although Putzeys & van de Walle (257) were not able to correlate electrophoretic mobilities of serum albumin and hemocyanin with variations in acidity and ionic strength of the solutions, Adair & Adair (258) found that the calculated mobilities of the ions of hemoglobin were in close agreement with the observed values, while Tiselius & Svensson (259) reported that the reduction in mobility caused by increasing ionic strength could be accounted for by the Debye-Hückel-Henry theory of electrophoresis. Studies have been made of the electrophoretic behavior of egg albumin partially digested with crystalline pepsin (259a); of microscopic particles in the presence of blood serum (260); of egg white over a range of pH values (261); and of crystalline pepsin (262). The

isoelectric point of secalin, the prolamine of rye, was shown to be at pH 6.67 by an electrophoretic mobility procedure (263).

The investigations of Fricke *et al.* (264) on the dielectric properties of amino acid solutions and gelatin-water systems have been continued. The relation of dielectric constant to molar concentration of amino acid was shown to be approximately linear while the dielectric absorption increased in proportion to the square of the frequency. In gelatin systems the dielectric constant was found to increase as the frequency decreased. It was concluded that the high polarizability of gelatin systems is not conditioned by the high moment of the gelatin molecule but is a property of the polymolecular layer of loosely bound water at the internal surfaces. The electric moments of serum albumin, serum pseudoglobulin-γ, and edestin were found by Oncley (265) to be about 380, 1200, and 1400 Debye units, respectively.

Structure.—Current assumptions concerning the structural and chemical characteristics of proteins have been discussed in preceding volumes of this *Review* (4, 76, 266, 267). It seems probable that proteins are intramolecular, three-dimensional systems of polypeptide chains held together in folded, cyclic, or cage-like structures by side-chain bridges of hydrogen bonds. The individual chains are considered to be made up of structurally related amino acid residues which occur in definite number and sequence. Complete details are lacking concerning the precise composition and structure of any protein but all proteins are thought to be constructed of amino acid residues and polypeptide chains bound together according to similar patterns. The denaturation, step-wise increase in molecular weight, specificity towards enzymes, and other properties of proteins appear to be explained by these assumptions.

Wrinch's theory that hexa-rings of proteins are linked to cyclical complexes by virtue of the tautomerism of the —CO—NH— and —C(OH)—N— bonds is, according to Fodor (268), similar to the latter author's hypothesis, first recorded in 1927, of the binding forces between closed polypeptide complexes, called "acropeptides." Interatomic distances in silk fibroin, α- and β-keratin, blood fibrin, stretched and unstretched hair, glycine and diketopiperazine crystals, and an extended polypeptide chain are given in a review by Corey (269). From investigations of the space requirements and orientation of amino acid residues in models of α-keratin and "supercontracted" keratin, Neurath (270) has concluded that fully extended polypeptide

chains can account for the observed properties and surface areas of protein monolayers but that neither hexagonal rings, either of the diamond or camphor type, nor the cyclol structure can accommodate the side chains of folded structures unless unreasonable distortion of bond angles are assumed. It was suggested that a spiral folding of the main polypeptide chains would permit alternate orientation of side chains, thus avoiding steric difficulties. Buswell *et al.* (271) have shown from infrared absorption studies that intermolecular NH—O, but not NH—N, bonds with absorption near 3.0 μ are present in N-substituted amides, peptides, and proteins. Since the coplanar configuration of the amide group must also be present in the peptide chain, cis-trans isomerism of hydrogen atoms is a variable to be considered in explaining the denaturation of proteins. The number of co-ordinate water molecules potentially possible in a 35 per cent water–65 per cent gelatin system has been computed by Sponsler *et al.* (272) to be approximately 800 to 850. It was assumed that the various atomic groups containing oxygen and nitrogen atoms are hydration centers, that the gelatin chain contains 288 amino acid residues and has a molecular weight of 27,000, and that only about 750 water molecules per gelatin molecule are present. The difference between the estimated and actual number of water molecules was assumed to be explained by a protein-to-protein cohesion through bridges between $=C=O$ and $=NH$ groups. Extensive discussions of the structural features of proteins and protein gels have been presented in reviews by Myers & France (273), Weidinger (274), Kruyt (275), Braybrooks (276), Vasiliu (277), and Pauling (278). The last author has presented, also, an interesting theory of the structure and process of formation of antibodies.

Miscellaneous papers.—Viscosity-fluid relations of proteins (279); an active allergenic protein component of cottonseed (280); ultracentrifugation studies on tobacco mosaic and bushy stunt virus (281); heat capacities of *dl*-citrulline, *dl*-ornithine, *l*(-)-proline, and taurine (282); preparation and properties of serum and plasma proteins (283, 284, 285); physical properties of alfalfa mosaic virus (286); the spreading number of proteins (287); viscosity of surface layers of proteins (288); electrokinetic aspects of surface chemistry (289, 290); use of electrophoresis in biological problems (291); determination of threonine in proteins (292); sulfur distribution in tobacco mosaic virus protein (293); dielectric constants of gelatin fractions (294); electroosmosis in gelatin (295); structure of gelatin sols and gels (296); diffraction of x-rays by gelatin gels (297); osmotic relations between egg white and egg yolk (298); properties of hemocyanins (299); elastic properties of ovokeratin (300); characteristics of autoclaved and enzymatic hydrolyzates of gelatin (301); electroreduction of cyclic and

open-chain peptides (302); effect of foam-producing substances on proteins (303); soybean protein dispersions in formaldehyde solutions (304); molecular weight of egg albumin (305); nature of gluten protein (306); apparent molecular shape and molecular weight of proteins (307); viscosity and shape of protein molecules (308); influence of salts on the melting point of gelatin sols (309); equilibrium between calcium and purified globulin (310); effect of egg albumin on the solubility of thallous salts (311); denaturation of sericin (312); hydrothermal stability of corium proteins in combination with chromic salts (313); solubility of cottonseed proteins in alkaline solutions of neutral salts (314); combination of gelatin (315) and wool protein (316) with acids and bases; colloidochemical hydrolysis of proteins (317); optical rotation of glutamic acid from necrotic cells (318); γ-rayed arginine (319); polarographic-ultraviolet ray investigations of proteins (320); biuret complex from proteins (321); tobacco mosaic virus studies (322); comparison of properties of casein from different animals (323); photometry of proteins (324); chemical studies on hemoglobin (325), methemoglobin (326), and blood proteins (327); and fluorescence associated with proteins (328).

LITERATURE CITED

1. CLOUGH, G. W., *J. Chem. Soc.,* **113,** 526 (1918)
2. CLOUGH, G. W., *J. Chem. Soc.,* **107,** 1509 (1915)
3. RIMINGTON, C., *Ann. Rev. Biochem.,* **5,** 117 (1936)
4. BERGMANN, M., AND NIEMANN, C., *Ann. Rev. Biochem.,* **7,** 99 (1938)
5. LLOYD, D. J., AND SHORE, A., *Chemistry of the Proteins,* pp. 35–40 (P. Blakiston's Son and Company, Inc., Philadelphia, 1938)
6. CLARKE, H. T., in GILMAN, H., *Organic Chemistry, an Advanced Treatise,* II, 859 (John Wiley and Sons, Inc., New York, 1938)
7. KARRER, P., *Organic Chemistry,* p. 272 (Nordemann Publishing Company, New York, 1938)
8. GREENBERG, D. M., in SCHMIDT, C. L. A., *Chemistry of the Amino Acids and Proteins,* p. 552 (C. C. Thomas, Springfield, Illinois, 1938)
9. FREUDENBERG, K., AND RHINO, F., *Ber.,* **57,** 1547 (1924)
10. FREUDENBERG, K., AND MEISTER, M., *Ann.,* **518,** 86 (1935)
11. FISCHER, E., AND RASKE, K., *Ber.,* **40,** 3717 (1907)
12. FISCHER, E., AND RASKE, K., *Ber.,* **41,** 893 (1908)
13. SCHNEIDER, F., *Ann.,* **529,** 1 (1937)
14. LANGENBECK, W., *Ber.,* **58,** 227 (1925)
15. LEVENE, P. A., AND MARDESHEW, S., *J. Biol. Chem.,* **117,** 179 (1937)
16. WASER, E., AND BRAUCHLI, E., *Helv. Chim. Acta,* **7,** 740 (1924)
17. KARRER, P., AND ITSCHNER, V., *Helv. Chim. Acta,* **18,** 782 (1935)
18. KARRER, P., JÄGGI, W., AND TAKAHASHI, T., *Helv. Chim. Acta,* **8,** 360 (1925)
19. KARRER, P., ESCHER, K., AND WIDMER, R., *Helv. Chim. Acta,* **9,** 301 (1926)
20. CANZANELLI, A., HARINGTON, C. R., AND RANDALL, S. S., *Biochem. J.,* **28,** 68 (1934)
21. BARROW, F., AND FERGUSON, G. W., *J. Chem. Soc.,* 410 (1935)
22. LUTZ, O., AND JIRGENSONS, B., *Ber.,* **63,** 448 (1930)
23. LUTZ, O., AND JIRGENSONS, B., *Ber.,* **64,** 1221 (1931)
24. DAKIN, H. D., *Biochem. J.,* **13,** 398 (1919)
25. DUNN, M. S., AND STODDARD, M. P. (Unpublished data)
26. ABDERHALDEN, E., AND ZEISSET, W., *Z. physiol. Chem.,* **196,** 121 (1931)
27. ABDERHALDEN, E., FROEHLICH, C., AND FUCHS, D., *Z. physiol. Chem.,* **86,** 454 (1913)
28. FISCHER, E., AND JACOBS, W. A., *Ber.,* **39,** 2942 (1906)
29. FISCHER, E., *Ber.,* **39,** 2320 (1906)
30. MEYER, C. E., AND ROSE, W. C., *J. Biol. Chem.,* **115,** 721 (1936)
31. CADDEN, J. F., *Proc. Soc. Exptl. Biol. Med.,* **45,** 224 (1940)
32. ECK, J. C., AND MARVEL, C. S., *Org. Syntheses,* **19,** 18, 20, 61 (1939)
33. HERBST, R. M., AND SHEMIN, D., *Org. Syntheses,* **19,** 1, 4, 67 (1939)
34. GILLESPIE, H. B., AND SNYDER, H. R., *Org. Syntheses,* **14,** 80 (1934)
35. CARTER, H. E., AND WEST, H. D., *Org. Syntheses,* **20,** 81 (1940)
36. CARTER, H. E., AND WEST, H. D., *Org. Syntheses,* **20,** 101 (1940)
37. MARVEL, C. S., *Org. Syntheses,* **20,** 106 (1940)
38. WOOD, J. L., AND VIGNEAUD, V. DU, *J. Biol. Chem.,* **134,** 413 (1940)

39. MAYEDA, S., TURUMI, M., AND SUZUKI, T., *Bull. Inst. Phys. Chem. Research (Tokyo)*, **19**, 267 (1940)

40. MITRA, S. K., *J. Indian Chem. Soc.*, **7**, 799 (1930)

41. WEST, H. D., KRUMMEL, G. S., AND CARTER, H. E., *J. Biol. Chem.*, **122**, 605, (1938)

42. WEST, H. D., AND CARTER, H. E., *J. Biol. Chem.*, **122**, 611 (1938)

43. CARTER, H. E., HANDLER, P., AND MELVILLE, D. B., *J. Biol. Chem.*, **129**, 359 (1939)

44. McILWAIN, H., AND RICHARDSON, G. M., *Biochem. J.*, **33**, 44 (1939)

45. ADKINS, H., AND REEVE, E. W., *J. Am. Chem. Soc.*, **60**, 1328 (1938)

46. REDEMANN, C. E., AND DUNN, M. S., *J. Biol. Chem.*, **130**, 341 (1939)

47. PAINTER, E. P., *J. Am. Chem. Soc.*, **62**, 232 (1940)

48. HARINGTON, C. R., AND RANDALL, S. S., *Biochem. J.*, **25**, 1917 (1931)

49. FEOFILAKTOV, V., AND ONISHCHENKO, A., *Compt. rend. acad. sci. U.R.S.S.*, **20**, 133 (1938); *Chem. Abstracts*, **33**, 1725 (1939)

50. MARVEL, C. S., AND STODDARD, M. P., *J. Org. Chem.*, **3**, 198 (1938);

51. ARNOW, L. E., AND OPSAHL, J. C., *J. Biol. Chem.*, **134**, 649 (1940); DUNN, M. S., AND STODDARD, M. P., *J. Biol. Chem.*, **121**, 521 (1937)

52. ADAMSON, D. W., *J. Chem. Soc.*, 1564 (1939)

53. ABDERHALDEN, E., AND HEYNS, K., *Ber.*, **67**, 530 (1934)

54. ABDERHALDEN, E., *Z. physiol. Chem.*, **251**, 164 (1938)

55. DARAPSKY, A., *J. prakt. Chem.*, **146**, 250 (1936)

56. GAUDRY, R., AND KING, F. E. (Personal communication, April, 1940)

57. HAHN, D. A., McLEAN, M. J., AND ENDICOTT, M. M., *J. Am. Chem. Soc.*, **62**, 1087 (1940)

58. ASPINALL, S. R., *J. Am. Chem. Soc.*, **62**, 1202 (1940)

59. CURTIUS, T., AND SIEBER, W., *Ber.*, **55**, 1543 (1922); CURTIUS, T., *J. prakt. Chem.*, **70**, 57 (1904); *J. prakt. Chem.*, **26**, 145 (1882)

60. MEYER, K. H., AND GO, Y., *Helv. Chim. Acta*, **17**, 1488 (1934)

61. FRANKEL, M., AND KATCHALSKI, E., *Nature*, **144**, 330 (1939); FRANKEL, M., NEUFELD, O., AND KATCHALSKI, E., *Nature*, **144**, 832 (1939); *Nature*, **144**, 551 (1939)

62. PACSU, E., *J. Franklin Inst.*, **230**, 132 (1940)

63. JACKSON, R. W., AND CAHILL, W. M., *J. Biol. Chem.*, **126**, 37 (1938)

64. CAHILL, W. M., AND BURTON, I. F., *J. Biol. Chem.*, **132**, 161 (1940)

65. BERGMANN, M., AND STERN, F., *Ber.*, **63**, 437 (1930)

66. DUNN, M. S., in SCHMIDT, C. L. A., *Chemistry of the Amino Acids and Proteins*, p. 21 (C. C. Thomas, Springfield, Illinois, 1938)

67. LUDWIG, W., AND MUTZENBECHER, P. VON, *Z. physiol. Chem.*, **244**, IV (1936); **258**, 195 (1939)

68. MUTZENBECHER, P. VON, *Z. physiol. Chem.*, **261**, 253 (1939)

69. HARINGTON, C. R., AND RIVERS, R. V. P., *Nature*, **144**, 205 (1939)

70. BLOCK, JR., P., *J. Biol. Chem.*, **135**, 51 (1940)

71. HARINGTON, C. R., AND BARGER, G., *Biochem. J.*, **21**, 169 (1927)

72. SCHOENHEIMER, R., *Harvey Lect.*, 122 (1936–37)

73. SCHOENHEIMER, R., AND RITTENBERG, D., *Science*, **87**, 221 (1938); *Physiol. Rev.*, **20**, 218 (1940)

74. VERZÁR, F., *Ann. Rev. Biochem.*, **7**, 163 (1938); *Ann. Rev. Biochem.*, **8**, 249 (1939)

75. JACKSON, R. W., AND CHANDLER, J. P., *Ann. Rev. Biochem.*, **8**, 249 (1939)

76. HITCHCOCK, D. I., *Ann. Rev. Biochem.*, **9**, 173 (1940)

77. Lewis, H. B., and Garner, R. L., *Ann. Rev. Biochem.*, **9**, 277 (1940)
78. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **111**, 163 (1935)
79. Thompson, Jr., A. F., and Cromwell, N. H., *J. Am. Chem. Soc.*, **61**, 1374 (1939)
80. Vigneaud, V. du, Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **131**, 273 (1939)
81. Clutton, R. F., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.* **132**, 227 (1940)
82. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **134**, 785 (1940)
83. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 633 (1940)
84. Vigneaud, V. du, Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940)
85. Vigneaud, V. du, Dyer, H. M., and Harmon, J., *J. Biol. Chem.*, **101**, 719 (1933)
86. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939)
87. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130**, 703 (1939)
88. Ratner, S., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **134**, 653 (1940)
89. Moss, A. R., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 415 (1940)
90. Ratner, S., Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 357 (1940)
91. Rittenberg, D., Ratner, S., and Hoberman, H. D., *J. Am. Chem. Soc.*, **62**, 2249 (1940)
92. Clarke, H. T., *Org. Syntheses*, **20**, 23 (1940)
93. Fox, S. W., *J. Biol. Chem.*, **123**, 687 (1938)
94. Cortese, F., *Org. Syntheses*, **18**, 77 (1938)
95. Greenstein, J. P., *J. Org. Chem.*, **2**, 480 (1937–38)
96. Harvey, D. G., and Robson, W., *J. Chem. Soc.*, 97 (1938)
97. Schöberl, A., and Braun, H., *Ann.*, **542**, 274 (1939)
98. Nagase, Y., *J. Pharm. Soc. Japan*, **58**, 185 (1938)
99. Weinstock, Jr., H. H., and May, E. L., *J. Am. Chem. Soc.*, **62**, 3266 (1940)
100. Wood, J. L., and Vigneaud, V. du, *J. Biol. Chem.*, **130**, 109 (1939)
101. Vigneaud, V. du, and Patterson, W. I., *J. Biol. Chem.*, **109**, 97 (1935)
102. Fruton, J. S., Irving, Jr., G. W., and Bergmann, M., *J. Biol. Chem.*, **133**, 703 (1940)
103. Behrens, O. K., Doherty, D. G., and Bergmann, M. *J. Biol. Chem.*, **136**, 61 (1940)
104. Pacsu, E., and Mullen, 2nd, J. W., *J. Biol. Chem.*, **136**, 335 (1940)
105. Fischer, E., *Ber.*, **32**, 2451 (1899)
106. Pope, W. J., and Gibson, C. S., *J. Chem. Soc.*, **101**, 939 (1912)
107. Fischer, E., and Skita, A., *Z. physiol. Chem.*, **33**, 177 (1901)
108. Fieser, L. F., and Creech, H. J., *J. Am. Chem. Soc.*, **61**, 3502 (1939)
109. Zanetti, J. E., and Bashour, J. T., *J. Am. Chem. Soc.*, **62**, 1511 (1940)
110. Wood, J. L., and Fieser, L. F., *J. Am. Chem. Soc.*, **62**, 2674 (1940)
111. Carter, H. E., and Melville, D. B., *J. Biol. Chem.*, **133**, 109 (1940)
112. Reichstein, T., and Grüssner, A., *Helv. Chim. Acta*, **23**, 650 (1940)
113. Klemme, C. J., and Hunter, J. H., *J. Org. Chem.*, **5**, 227, 508 (1940)

114. APPEL, W., *Z. physiol. Chem.*, **260**, 148 (1939)
115. CARTER, H. E., HANDLER, P., AND STEVENS, C. M., *J. Biol. Chem.*, **133**, xviii (1940)
116. TOENNIES, G., *J. Biol. Chem.*, **132**, 455 (1940); **133**, cii (1940)
117. OVAKIMIAN, G., CHRISTMAN, C. C., KUNA, M., AND LEVENE, P. A., *J. Biol. Chem.*, **134**, 151, (1940)
118. OVAKIMIAN, G., KUNA, M., AND LEVENE, P. A., *J. Biol. Chem.*, **135**, 91 (1940)
119. KENTTÄMAA, M., *Suomen Kemistilehti, A,* **12**, 109 (1939)
120. HOCH, H., *Biochem. J.,* **34**, 1209 (1940)
121. LECOQ, H., *Bull. soc. chim. biol.,* **22**, 112 (1940)
122. KEYS, A., *J. Biol. Chem.,* **132**, 181 (1940)
123. SULLIVAN, M. X., AND MCCARTHY, T. E., *J. Biol. Chem.,* **133**, c (1940)
124. KURIHARA, Z., *J. Biochem. (Japan),* **30**, 205 (1939)
125. RACKER, E., *Biochem. J.,* **34**, 89 (1940)
126. BRAND, E., AND KASSELL, B., *J. Biol. Chem.,* **131**, 489 (1939)
127. SHAW, J. L. D., AND MCFARLANE, W. D., *J. Biol. Chem.,* **132**, 387 (1940)
128. BLOCK, R. J., BOLLING, D., AND WEBB, M., *J. Biol. Chem.,* **133**, xiv (1940)
129. BERGMANN, M., AND STEIN, W. H., *J. Biol. Chem.,* **128**, 217 (1939)
130. ING, H. R., AND BERGMANN, M., *J. Biol. Chem.,* **129**, 603 (1939)
131. STEIN, W. H., AND BERGMANN, M., *J. Biol. Chem.,* **134**, 627 (1940)
132. DOHERTY, D. G., STEIN, W. H., AND BERGMANN, M., *J. Biol. Chem.,* **135**, 487 (1940)
133. REDEMANN, C. E., AND NIEMANN, C., *J. Am. Chem. Soc.,* **62**, 590 (1940)
134. BLOCK, R. J., *J. Biol. Chem.,* **133**, 67 (1940)
135. VICKERY, H. B., *J. Biol. Chem.,* **132**, 325 (1940)
136. RITTENBERG, D., AND FOSTER, G. L., *J. Biol. Chem.,* **133**, 737 (1940)
137. USSING, H. H., *Nature,* **144**, 977 (1939)
138. GRAFF, S., RITTENBERG, D., AND FOSTER, G. L., *J. Biol. Chem.,* **133**, 745 (1940)
139. ALBANESE, A. A., *J. Biol. Chem.,* **134**, 467 (1940)
140. KOLB, J. J., AND TOENNIES, G., *Ind. Eng. Chem., Anal. Ed.,* **12**, 723 (1940)
141. KENDRICK, A. B., AND HANKE, M. E., *J. Biol. Chem.,* **117**, 161 (1937)
142. KENDRICK, A. B., AND HANKE, M. E., *J. Biol. Chem.,* **132**, 739 (1940)
143. DUNN, M. S., AND PORUSH, I., *J. Biol. Chem.,* **127**, 261 (1939)
144. VAN SLYKE, D. D., AND FOLCH, J., *J. Biol. Chem.,* **136**, 509 (1940)
145. GORNALL, A. G., AND HUNTER, A., *Biochem. J.,* **34**, 192 (1940)
146. BAPTIST, N. G., AND ROBSON, W., *Biochem. J.,* **34**, 221 (1940)
147. BERGMANN, M., *J. Biol. Chem.,* **110**, 471 (1935)
148. MAYEDA, S., *Bull. Inst. Phys. Chem. Research (Tokyo),* **19**, 261, 1940); MAYEDA, S., AND MARUO, B., *Bull. Inst. Phys. Chem. Research (Tokyo),* **19**, 271 (1940)
149. VAN SLYKE, D. D., HILLER, A., MACFADYEN, D. A., HASTINGS, A. B., AND KLEMPERER, F. W., *J. Biol. Chem.,* **133**, 287 (1940)
150. DAMODARAN, M., AND RAMACHANDRAN, B. V., *Nature,* **145**, 857 (1940)
151. SUBBAROW, Y., AND HITCHINGS, G. H., *J. Biol. Chem.,* **133**, c (1940)
152. KUHN, R., AND WIELAND, T., *Ber.,* **73**, 962 (1940)
153. IMAMURA, H., *J. Biochem. (Japan),* **30**, 479 (1939)

154. FUKUDA, S., *J. Biochem. (Japan)*, **30**, 141 (1939)
155. CHIBNALL, A. C., REES, M. W., WILLIAMS, E. F., AND BOYLAND, E., *Biochem. J.*, **34**, 285 (1940)
156. DODDS, E. C., AND DICKENS, F., *Ann. Rev. Biochem.*, **9**, 423 (1940)
157. DITTMAR, C., *Z. Krebsforsch.*, **49**, 397, 441 (1939)
158. OTTAWA, H., *Z. Krebsforsch.*, **49**, 677 (1939)
159. JOHNSON, J. M., *J. Biol. Chem.*, **132**, 781 (1940); **134**, 459 (1940)
160. ARNOW, L. E., AND OPSAHL, J. C., *J. Biol. Chem.*, **133**, 765 (1940); *Science*, **90**, 257 (1939); CLARK, W. G., ARNOW, L. E., AND OPSAHL, J. C., *Proc. Soc. Exptl. Biol. Med.*, **43**, 767 (1940)
161. LIPMANN, F., BEHRENS, O. K., KABAT, E. A., AND BURK, D., *Science*, **91**, 21 (1940)
162. GRAFF, S., RITTENBERG, D., AND FOSTER, G. L., *J. Biol. Chem.*, **133**, 745 (1940)
163. CHASE, M. W., AND LANDSTEINER, K., *Ann., Rev. Biochem.*, **8**, 579 (1939)
164. FRÄNKEL, S., AND GALLIA, K., *Biochem. Z.*, **134**, 308 (1922)
165. JACOBS, W. A., AND CRAIG, L. C., *J. Biol. Chem.*, **110**, 521 (1935)
166. SMITH, S., AND TIMMIS, G. M., *J. Chem. Soc.*, 396 (1937)
167. SYNGE, R. L. M., *Biochem. J.*, **33**, 1913, 1918, 1924, 1931 (1939)
168. CALVERY, H. O., in SCHMIDT, C. L. A., *Chemistry of the Amino Acids and Proteins*, p. 123 (C. C. Thomas, Springfield, Illinois, 1938)
169. NICOLET, B. H., AND SHINN, L. A., *J. Am. Chem. Soc.*, **61**, 1615 (1939)
170. BLOCK, R. J., AND BOLLING, D., *J. Biol. Chem.*, **130**, 365 (1939)
171. MARTIN, A. J. P., AND SYNGE, R. L. M., *Nature*, **146**, 491 (1940)
171a. MARTIN, A. J. P., AND SYNGE, R. L. M. (Personal communication, December, 1940)
172. SEIKI, Y., *J. Biochem. (Japan)*, **30**, 107 (1939)
173. TOMITA, M., AND SEIKI, Y., *J. Biochem. (Japan)*, **30**, 101 (1939)
174. BERGMANN, M., AND LISSITZIN, M., *Ber.*, **63**, 310 (1930)
175. GOUBEAU, J., AND LÜNING, A., *Ber.*, **73**, 1053 (1940); WRIGHT, N., AND LEE, W. C., *Nature*, **139**, 551 (1937)
176. EDSALL, J. T., *J. Chem. Physics*, **4**, 1 (1936); **5**, 225, 508 (1937); *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 40 (1938)
177. EDSALL, J. T., AND SCHEINBERG, H., *J. Chem. Physics*, **8**, 520 (1940)
178. BAUR, E., *Helv. Chim. Acta*, **23**, 233 (1940)
179. SMITH, A. K., HANDLER, P., AND MRGUDICH, J. N., *J. Phys. Chem.*, **44**, 874 (1940)
180. LEVY, M., *J. Biol. Chem.*, **99**, 767 (1933); **109**, 365 (1935)
181. TOMIYAMA, T., *J. Biol. Chem.*, **111**, 51 (1935)
182. WADSWORTH, A., AND PANGBORN, M. C., *J. Biol. Chem.*, **116**, 423 (1936)
183. BATCHELDER, A. C., AND SCHMIDT, C. L. A., *J. Phys. Chem.*, **43**, 1121 (1939); **44**, 880, 893 (1940)
184. SMITH, E. R. B., AND SMITH, P. K., *J. Biol. Chem.*, **117**, 209 (1937); **132**, 47 (1940)
185. SMITH, P. K., AND SMITH, E. R. B., *J. Biol. Chem.*, **121**, 607 (1937); **132**, 57 (1940)
186. STURTEVANT, J. M., *J. Am. Chem. Soc.*, **62**, 1879 (1940)
187. GUCKER, JR., F. T., PICKARD, H. B., AND FORD, W. L., *J. Am. Chem. Soc.*, **62**, 2698 (1940)

188. PERTZOFF, V. A., *J. Am. Chem. Soc.*, **62**, 3150 (1940)
189. PERTZOFF, V. A., *Pouvoir rotatoire des ions de l'acide* d-*glutamique* (Imprimerie de la Charite, Montpellier, France, 1937)
190. KAUZMANN, W. J., WALTER, J. E., AND EYRING, H., *Chem. Rev.*, **26**, 339 (1940)
191. PARTS, A., *Pub. Tech. Univ. Estonia Tallinn, Ser. A*, No. 8, 3 (1939)
192. HILL, T. L., AND BRANCH, G. E. K., *Science*, **91**, 145 (1940)
193. MASLOV, A. T., *Bull. biol. méd. exptl. U.R.S.S.*, **4**, 411 (1937)
194. MEYER, K. H., AND KLEMM, O., *Helv. Chim. Acta*, **23**, 25 (1940)
195. OHIRA, T., *J. Agr. Chem. Soc. (Japan)*, **16**, 293 (1940)
196. KONIKOV, A. P., AND LERMAN, K. N., *J. Gen. Chem., (U.S.S.R.)*, **9**, 2115 (1939)
197. SMITH, E. R. B., AND SMITH, P. K., *J. Biol. Chem.*, **135**, 273 (1940)
198. LINDERSTRØM-LANG, K., AND JACOBSEN, C. F., *Enzymologia*, **8**, 64 (1940)
199. HUGGINS, M. L., *J. Am. Chem. Soc.*, **61**, 2982 (1939)
200. VIGNEAUD, V. DU, MILLER, G. L., AND RODDEN, C. J., *J. Biol. Chem.*, **131**, 631 (1939)
201. ROMANS, R. G., SCOTT, D. A., AND FISHER, A. M., *Ind. Eng. Chem., Ind. Ed.*, **32**, 908 (1940)
202. ROUTH, J. I., *J. Biol. Chem.*, **135**, 175 (1940)
203. HARRIS, R. L., AND MATTILL, H. A., *J. Biol. Chem.*, **132**, 477 (1940)
204. RUTHERFORD, H. A., AND HARRIS, M., *J. Research Natl. Bur. Standards*, **24**, 415 (1940)
205. ROBINSON, H. W., AND HOGDEN, C. G., *J. Biol. Chem.*, **135**, 707, 727 (1940)
206. KOSOLAPOV, B. M., *J. Applied Chem. (U.S.S.R.)*, **13**, 314 (1940)
207. KIHARA, K., *J. Agr. Chem. Soc. (Japan)*, **16**, 206 (1940)
208. LAINE, T., *Suomen Kemistilehti, B*, **12**, 23 (1939)
209. CLAY, R. C., COOK, K., AND ROUTH, J. I., *J. Am. Chem. Soc.*, **62**, 2709 (1940)
210. BRIGGS, D. R., *J. Biol. Chem.*, **134**, 261 (1940)
211. BRAND, E., AND KASSELL, B., *J. Biol. Chem.*, **133**, 437 (1940)
212. CREECH, H. J., AND JONES, R. N., *J. Am. Chem. Soc.*, **62**, 1970 (1940)
213. JUNG, F., *Biochem. Z.*, **304**, 37 (1940)
213a. MILLER, C. O., AND ROBERTS, R. G., *J. Am. Chem. Soc.*, **61**, 3554 (1939)
214. ROSNER, L., *J. Biol. Chem.*, **132**, 657 (1940)
215. GUREVICH, D. I., *J. Physiol. (U.S.S.R.)*, **27**, 615 (1939)
216. TAYLOR, A. R., SHARP, D. G., FINKELSTEIN, H., BEARD, D., AND BEARD, J. W., *Proc. Soc. Exptl. Biol. Med.*, **43**, 648 (1940)
217. PFANKUCH, E., *Biochem. Z.*, **303**, 342 (1940)
218. PFANKUCH, E., KAUSCHE, G. A., AND STUBBE, H., *Biochem. Z.*, **304**, 238 (1940)
219. STANLEY, W. M., *Rept. Proc. Third Intern. Congr. Microbiol., 1939*, 43 (1940) ; *Ann. Rev. Biochem.*, **9**, 545 (1940)
220. NORTHEN, H. T., *Biodynamica*, **3**, 10 (1940) ; *Trans. Am. Microscop. Soc.*, **59**, 279 (1940) ; NORTHEN, H. T., AND MACVICAR, R., *Biodynamica*, **3**, 28 (1940)

221. RIMINGTON, C., AND VAN DEN ENDE, M., *Biochem. J.,* **34,** 941 (1940)
222. RIMINGTON, C., *Biochem. J.,* **34,** 931 (1940)
223. KONDO, K., AND SARATA, U., *J. Agr. Chem. Soc. (Japan),* **16,** 227, 271 (1940)
224. KUHN, R., SÖRENSEN, N. A., AND BIRKOFER, L., *Ber.,* **73,** 823 (1940)
225. TRISTRAM, G. R., *Biochem. J.,* **34,** 301 (1940)
226. KADATU, M., *Bull. Agr. Chem. Soc. (Japan),* **16,** 16 (1940)
227. KONDO, K., SINANO, S., AND MITUTA, H., *J. Agr. Chem. Soc. (Japan),* **16,** 233 (1940)
228. KONDO, K., HAYASI, S., AND MORISIGE, S., *J. Agr. Chem. Soc. (Japan),* **15,** 727 (1939); *Chem. Abstracts,* **34,** 137 (1940)
229. KONDO, K., NAGASIMA, M., AND MORISIGE, S., *J. Agr. Chem. Soc. (Japan),* **15,** 737 (1939); *Chem. Abstracts,* **34,** 120 (1940)
230. KONDO, K., AND MITUTA, H., *J. Agr. Chem. Soc. (Japan),* **16,** 159 (1940)
231. ASTRUP, T., AND DARLING, S., *J. Biol. Chem.,* **133,** 761 (1940)
232. DYCKERHOFF, H., AND GIGANTE, D., *Biochem. Z.,* **304,** 334 (1940)
233. BICKEL, A., AND PARLOW, A., *Biochem. Z.,* **304,** 105 (1940)
234. HIGHBERGER, J. H., AND O'FLAHERTY, F., *J. Intern. Soc. Leather Trades' Chem.,* **23,** 549 (1939)
235. TYLER, A., AND FOX, S. W., *Biol. Bull.,* **79,** 153 (1940)
236. FEDOTOVA, A. M., *Bull. biol. méd. exptl., U.R.S.S.,* **4,** 400 (1937)
237. NEDVED, M., AND WOLDRICHOVA, O., *Le Sang,* **13,** 972 (1939)
238. KINGSLEY, G. R., *J. Biol. Chem.,* **133,** 731 (1940)
239. MURRILL, W. A., BLOCK, W. D., AND NEWBURGH, L. H., *J. Biol. Chem.,* **133,** 521 (1940)
240. BAILEY, K., *Nature,* **145,** 934 (1940)
241. GREENSTEIN, J. P., AND EDSALL, J. T., *J. Biol. Chem.,* **133,** 397 (1940)
242. EDSALL, J. T., AND MEHL, J. W., *J. Biol. Chem.,* **133,** 409 (1940)
243. BULL, H. B., *J. Biol. Chem.,* **133,** 39 (1940)
244. CONN, J. B., KISTIAKOWSKY, G. B., AND ROBERTS, R. M., *J. Am. Chem. Soc.,* **62,** 1895 (1940)
245. ROCHE, J., AND CHOUAÏECH, M.-S., *Compt. rend.,* **209,** 1017 (1939); *Chem. Abstracts,* **34,** 2868 (1940)
246. ROCHE, J., AND CHOUAÏECH, M.-S., *Compt. rend. soc. biol.,* **133,** 474 (1940)
247. LANGMUIR, I., AND WAUGH, D. F., *J. Am. Chem. Soc.,* **62,** 2771 (1940)
248. CADWALLADER, C. J., HOWITT, F. O., AND SMITH, S. G., *Nature,* **146,** 301 (1940)
249. SALCHINKIN, A. P., *Colloid J. (U.S.S.R.),* **5,** 467 (1939)
250. MACPHERSON, C. F. C., AND HEIDELBERGER, M., *Proc. Soc. Exptl. Biol. Med.,* **43,** 646 (1940)
251. VOLKOV, E. N., AND DVINYANINOVA, I. L., *J. Applied Chem. (U.S.S.R.),* **13,** 267 (1940)
252. EBBECKE, U., *Biochem. Z.,* **304,** 177 (1940)
253. WANNOW, H. A., *Kolloid-Beihefte,* **50,** 367 (1939)
254. SPIEGEL-ADOLF, M., AND HENNY, G. C., *J. Biol. Chem.,* **133,** xciv (1940)
255. TISELIUS, A., *Rept. Proc. 3rd Intern. Congr. Microbiol., 1939,* 54 (1940); *J. Franklin Inst.,* **228,** 797 (1939)
256. ABRAMSON, H. A., *Trans. Faraday Soc.,* **36,** 5 (1940)

257. PUTZEYS, P., AND VAN DE WALLE, P., *Trans. Faraday Soc.*, 36, 32 (1940)
258. ADAIR, G. S., AND ADAIR, M. E., *Trans. Faraday Soc.*, 36, 23 (1940)
259. TISELIUS, A., AND SVENSSON, H., *Trans. Faraday Soc.*, 36, 16 (1940)
259a. TISELIUS, A., AND ERIKSSON-QUENSEL, I.-B., *Biochem. J.*, 33, 1752 (1939)
260. MOYER, L. S., AND MOYER, E. Z., *J. Biol. Chem.*, 132, 373 (1940)
261. LONGSWORTH, L. G., CANNAN, R. K., AND MACINNES, D. A., *J. Am. Chem. Soc.*, 62, 2580 (1940)
262. HERRIOTT, R. M., DESREUX, V., AND NORTHROP, J. H., *J. Gen. Physiol.*, 23, 439 (1940)
263. ANDREWS, A. C., *J. Am. Chem. Soc.*, 62, 942 (1940)
264. FRICKE, H., AND PARTS, A., *J. Phys. Chem.*, 42, 1171 (1938) ; FRICKE, H., AND JACOBSON, L. E., *J. Phys. Chem.*, 43, 781 (1939) ; FRICKE, H., AND PARKER, E., *J. Phys. Chem.*, 44, 716 (1940)
265. ONCLEY, J. L., *J. Phys. Chem.*, 44, 1103 (1940)
266. ASTBURY, W. T., *Ann. Rev. Biochem.*, 8, 113 (1939)
267. TISELIUS, A., *Ann. Rev. Biochem.*, 8, 155 (1939)
268. FODOR, A., *Enzymologia*, 6, 207 (1939)
269. COREY, R. B., *Chem. Rev.*, 26, 227 (1940)
270. NEURATH, H., *J. Phys. Chem.*, 44, 296 (1940)
271. BUSWELL, A. M., DOWNING, J. R., AND RODEBUSH, W. H., *J. Am. Chem. Soc.*, 62, 2759 (1940) ; BUSWELL, A. M., KREBS, K. F., AND RODEBUSH, W. H., *J. Phys. Chem.*, 44, 1126 (1940)
272. SPONSLER, O. L., BATH, J. D., AND ELLIS, J. W., *J. Phys. Chem.*, 44, 996 (1940)
273. MYERS, W. G., AND FRANCE, W. G., *J. Phys. Chem.*, 44, 1113 (1940)
274. WEIDINGER, A., *Collegium*, 1 (1940)
275. KRUYT, H. R., *Chimie & industrie*, 42, 587 (1939)
276. BRAYBROOKS, W. E., *J. Intern. Soc. Leather Trades' Chem.*, 23, 515 (1939)
277. VASILIU, H., *Bul. Facultăt. Ştiinţe Agr. Chişinău, Comun. Lab. Chim. Agr.*, 3, 53 (1940)
278. PAULING, L., *J. Am. Chem. Soc.*, 62, 2643 (1940)
279. TREFFERS, H. P., *J. Am. Chem. Soc.*, 62, 1405 (1940)
280. SPIES, J. R., COULSON, E. J., BERNTON, H. S., AND STEVENS, H., *J. Am. Chem. Soc.*, 62, 1420 (1940)
281. LAUFFER, M. A., *J. Phys. Chem.*, 44, 1137 (1940)
282. HUFFMAN, H. M., AND FOX, S. W., *J. Am. Chem. Soc.*, 62, 3464 (1940)
283. COHN, E. J., MCMEEKIN, T. L., ONCLEY, J. L., NEWELL, J. M., AND HUGHES, W. L., *J. Am. Chem. Soc.*, 62, 3386 (1940)
284. MCMEEKIN, T. L., *J. Am. Chem. Soc.*, 62, 3393 (1940)
285. COHN, E. J., LUETSCHER, JR., J. A., ONCLEY, J. L., ARMSTRONG, JR., S. H., AND DAVIS, B. D., *J. Am. Chem. Soc.*, 62, 3396 (1940)
286. LAUFFER, M. A., AND ROSS, A. F., *J. Am. Chem. Soc.*, 62, 3296 (1940)
287. DERVICHIAN, D. G., *Nature*, 144, 629 (1939)
288. JOLY, M., *J. chim. phys.*, 36, 285 (1939)
289. MOYER, L. S., AND MOYER, E. Z., *J. Biol. Chem.*, 132, 357, 373 (1940)
290. MOYER, L. S., AND GORIN, M. H., *J. Biol. Chem.*, 133, 605 (1940)
291. MOYER, L. S., *Trans. Faraday Soc.*, 36, 248 (1940)
292. HIGASI, T., MAYEDA, S., AND MATUOKA, H., *Bull. Inst. Phys. Chem. Research (Tokyo)*, 17, 1292 (1938)

293. Ross, A. F., *J. Biol. Chem.,* **136,** 119 (1940)
294. Lipatov, S. M., and Shul'man, M. S., *Colloid J. (U.S.S.R.),* **5,** 555 (1939)
295. Swyngedauw, J., *Kolloid-Z.,* **88,** 318 (1939)
296. Sheppard, S. E., Houck, R. C., and Dittmar, C., *J. Phys. Chem.,* **44,** 185 (1940)
297. Barnes, W. H., and Matthews, E. W., *Biodynamica,* No. 49, 1 (1939)
298. Basu, N. M., and Mitra, M. C., *J. Indian Chem. Soc.,* **17,** 111 (1940)
299. Roche, J., and Derrien, Y., *Bull. soc. chim. biol.,* **22,** 7 (1940)
300. Baudouy, C.-T., *J. chim. phys.,* **36,** 41 (1939)
301. Gavrilov, N. I., and Balabukha-Poptsova, V. S., *Enzymologia,* **7,** 245 (1939)
302. Gavrilov, N. I., and Koperina, A. V., *J. Gen. Chem. (U.S.S.R.),* **9,** 1394 (1939); *Chem. Abstracts,* **34,** 1615 (1940)
303. Kuhn, R., and Bielig, H.-J., *Ber.,* **73,** 1080 (1940)
304. Smith, A. K., and Max, H. J., *Ind. Eng. Chem., Ind. Ed.,* **32,** 411 (1940)
305. Bernhart, F. W., *J. Biol. Chem.,* **132,** 189 (1940)
306. McCalla, A. G., and Gralén, N., *Nature,* **146,** 60 (1940)
307. Neurath, H., and Cooper, G. R., *J. Am. Chem. Soc.,* **62,** 2248 (1940)
308. Mehl, J. W., Oncley, J. L., and Simha, R., *Science,* **92,** 132 (1940)
309. Merckel, J. H. C., and Haayman, P. W., *Kolloid-Z.,* **87,** 59 (1939)
310. Drinker, N., Green, A. A., and Hastings, A. B., *J. Biol. Chem.,* **131,** 641 (1939)
311. Eversole, W. G., and Bjork, C. F., *Proc. Iowa Acad. Sci.,* **46,** 200 (1939)
312. Hirose, Z., *J. Agr. Chem. Soc. (Japan),* **16,** 209 (1940)
313. Gustavson, K. H., *Svensk Kem. Tid.,* **52,** 75 (1940)
314. Olcott, H. S., and Fontaine, T. D., *J. Am. Chem. Soc.,* **62,** 1334 (1940)
315. Friedman, L., Klemm, K., and Thompson, F. K., *J. Phys. Chem.,* **43,** 1133 (1939)
316. Steinhardt, J., and Harris, M., *J. Research Natl. Bur. Standards,* **24,** 335 (1940)
317. D'yachkovskiĭ, S. I., Tumanovskaya, G. V., and Rabinovich, I. B., *Colloid J. (U.S.S.R.),* **5,** 501 (1939)
318. Bayerle, H., and Borger, G., *Biochem. Z.,* **305,** 418 (1940)
319. van Heys, F. W., *Biochem. Z.,* **305,** 422 (1940)
320. Schmidt, H. W., *Biochem. Z.,* **306,** 167 (1940)
321. Küntzel, A., and Dröscher, T., *Biochem. Z.,* **306,** 177 (1940)
322. Pfankuch, E., *Biochem. Z.,* **306,** 125 (1940); Pfankuch, E., and Kausche, G. A., *Biochem. Z.,* **306,** 68 (1940)
323. Kóvacs, E., *Biochem. Z.,* **306,** 74 (1940)
324. Jendrassik, L., and Polgár, A., *Biochem. Z.,* **305,** 237 (1940)
325. Jung, F., *Biochem. Z.,* **305,** 248 (1940)
326. Hecht, G., *Biochem. Z.,* **305,** 290 (1940)
327. Bálint, P., and Bálint, M., *Biochem. Z.,* **305,** 310 (1940)
328. Reeder, W., and Nelson, V. E., *Proc. Soc. Exptl. Biol. Med.,* **45,** 792 (1940)

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THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR

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Circumstances beyond the control of the reviewer have made it impossible to consider all the publications dealing with the various aspects of the chemistry and metabolism of the compounds of sulfur. A number of journals could not be obtained because of the conflict abroad. Although abstracts have been available, reference has been made in this review only to publications which have been seen in full. Restriction of space has made it necessary to limit the number of papers reviewed. It is highly regrettable that this has prevented discussion of many interesting aspects of sulfur, e.g., certain sulfur compounds other than amino acids (thiocyanates, thiourea), sulfur metabolism in plants, sulfur requirements of bacteria, chemistry of toxins, sulfur-containing chemotherapeutic agents, proliferation *in vitro,* and conjugated sulfuric acid esters of the type of heparin.

Organic chemistry and reactions.—Cysteine conjugates of certain of the polycyclic hydrocarbons have been synthesized and their properties studied (1). The condensation of α-amino-β-chloropropionic acid with the 5-mercaptan of 3,4-benzanthracene yields the corresponding S-cysteine compound. 1,2-Benzanthryl-10-methyl-S-*l*-cysteine was prepared by the interaction of 10-chloromethyl-1,2-benzanthracene with sodium cysteinate in liquid ammonia. The cysteine derivatives obtained are considerably more unstable than the corresponding conjugates with single aromatic nuclei, e.g., *p*-bromophenyl-S-cysteine, and decompose when warmed in neutral aqueous solution with the formation of disulfides.

A new synthesis of cystine has been described (2). Condensation of benzylmercaptan with formaldehyde, in the presence of hydrochloric acid gives benzylthiomethyl chloride; the latter is conjugated with phthalimidomalonic ester, and the resulting product hydrolyzed to give benzyl-*dl*-cysteine. Treatment with sodium in liquid ammonia results in the formation of optically inactive cysteine from which *dl*-cystine is obtained by oxidation. Although cystine is readily avail-

able from natural sources, this synthesis makes possible the introduction of any or all of the following isotopic elements into the cystine molecule: carbon, hydrogen, nitrogen, and sulfur.

Isocysteine and isocystine have been made available through a synthesis developed by Schöberl & Braun (3). Treatment of either α-bromo-β-alanine hydrobromide or α-chloro-β-alanine hydrochloride with sodium sulfide and reduction of the reaction product with tin and hydrochloric acid gives isocysteine hydrochloride. The latter is oxidized to isocystine. The labilizing effect of the amino group on the sulfur of the molecule has been studied. Boiling isocystine with water, or with N sulfuric acid, results in the formation of 17.5 and 25.7 per cent, respectively, of hydrogen sulfide, whereas dithiodiglycolic acid, under similar conditions, gives 6.1 and 0 per cent hydrogen sulfide, respectively. Comparative data for alkaline hydrolysis are also given.

Trideuteriomethionine has been prepared (4) by using trideuteriomethyliodide in the first step of the synthesis of methionine (5). In continuation of his studies of methionine, Toennies (6) has described the formation of crystalline sulfonium salts of methionine of the composition,

$$^{-}OOC \cdot CH(NH^{+}_{3}) \cdot CH_{2} \cdot CH_{2} \cdot S \cdot (CH_{3}) \cdot R^{+}X^{-}.$$
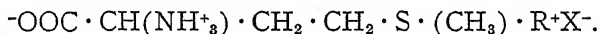
The isolated salts are water-soluble, neutral substances.

Clarke (7) has given conditions for producing cysteic acid monohydrate in 81 to 90 per cent yield by oxidation of cystine with bromine. S-Benzylglutathione was obtained (8) by condensation of glutathione with benzyl chloride in liquid ammonia solution. The hydrolysis of 2-mercaptothiazoline, or the action of hydrogen sulfide upon a dilute solution of ethylenimine, with subsequent oxidation of the mercaptan, results in the formation of cystamine (9). Condensation of the urea derivative of cystamine with malonic acid gives a disulfide barbituric acid which may be employed for the preparation of disulfides of certain pyrimidines and of uric acid. In a synthetic approach to compounds with possible oxytocic properties, the carbobenzoxy method has been employed for the synthesis of cysteinylcholine (10); oxidation of the latter yields the disulfide form.

During the course of a study of the reactions of proteins with ferricyanide, Anson (11) observed the formation of ferrocyanide from cysteine and ferricyanide over the wide pH range 3.0 to 9.6. The

oxidation is stoichiometric and is independent, within rather wide limits, of ferricyanide concentration. The same author (12) has examined the oxidation of cysteine by iodine at pH 3.2 and finds a consumption of three molecules of iodine per molecule of cysteine, corresponding to the formation of cysteic acid. Oxidation of cysteine and of thioglycolic acid to disulfide by 4-amino-2-methylnaphthol at pH 7.8 has been reported (13). The high vitamin-K activity of the latter compound adds interest to the findings in view of the fact that cysteine prolongs the clotting time of blood. However, the naphthol derivative had little effect on the oxidation of reduced glutathione, which also increases clotting time.

Several crystalline derivatives of the sulfur-containing amino acids have been described by investigators searching for new reagents for the isolation of amino acids. Doherty, Stein & Bergmann (14) have included *l*-cysteine, *l*-cystine, and *dl*-methionine among the amino acids used in a comprehensive study of the preparation and solubilities of the amino acid salts of a large number of aromatic sulfonic acids. Redemann & Niemann (15) have described the crystalline 5-nitrobarbiturates of *l*-cystine, *l*-cysteine, and *dl*-methionine. Solubilities of the salts in water at 25° are given. The picrolonate of *l*-methionine has been prepared (16).

From an analysis of the anodic waves of cysteine at the dropping mercury electrode in 0.1 M perchloric acid (pH 1.0), Kolthoff & Barnum (17) concluded that the compound formed is a mercurous cysteine, $Hg \cdot S \cdot CH_2 \cdot CHNH_2 \cdot COOH$. The anodic reaction is given by the equation: $R \cdot SH + Hg \leftrightarrows Hg \cdot SR + H^+ + e$. At the platinum electrode the anodic waves correspond to the formation of cystine from cysteine.

Newly isolated sulfur compounds.—The first occurrence of a sulfone in plant or animal tissues has been reported simultaneously by two laboratories. Dimethylsulfone has been isolated from beef blood (18) and from beef adrenals (19). Approximately one ton of dried blood yielded 3.5 gm. of sulfone. The yield from adrenal tissue was approximately one half of that from blood. Taurine and the potassium salt of ethyl hydrogen sulfate were also obtained from the adrenal extracts. The occurrence of dimethylsulfone in the adrenal gland is of considerable interest since bis-β-hydroxyethyl sulfoxide has been obtained from the same source (20).

In a preliminary communication, Koschara (21) has reported the isolation of 40 to 80 mg. of lemon-yellow crystals containing approxi-

mately 20 per cent sulfur from a thousand liters of human urine. The isolated substance was named urothion. It exhibits no melting point and is soluble in acids and alkali. Solutions of the compound give two characteristic tests: a ring test with sulfuric acid, similar to that given by thiophenols, and an olive-green fluorescence in the ultraviolet after oxidation of the sulfuric acid solution with potassium permanganate. Catalytic hydrogenation yields one mole of methyl mercaptan. Urothion contains an amino group (Van Slyke), and gives a tetraacetyl derivative. An empirical formula of $C_{11}H_{13}O_8N_5S_2$ is suggested. Urithion was found in human and cattle liver as well as in other organs. Its structure and physiological significance are unknown at the present time.

Analytical methods.—A careful study (22) of tetrahydroxyquinone as an indicator in the direct titration of sulfate ion by barium chloride has made available a useful volumetric method for the determination of inorganic sulfate. Sulfur may be estimated in amounts from 0.5 to 2.0 mg. Marenzi & Banfi (23) have described a method for the determination of benzidine sulfate colorimetrically, employing the weak reducing power of benzidine on phosphotungstomolybdic acid (Folin's phenol reagent). Sulfur distribution may be done in blood and urine, and procedures are given for removing interfering substances. Five µg. of sulfur can be estimated with an error of 3 per cent. Inorganic sulfate in tissue extracts and in biological fluids may also be determined by a nephelometric method (24). Although variations of from $+12$ to -10 per cent have been observed in recoveries of inorganic sulfate added to serum, the ease and speed with which the determinations may be made strongly recommend the method when large changes in sulfate concentrations are expected.

A micromethod (25) for the determination of small quantities of hydrogen sulfide in protein solutions utilizes a microapparatus for rapidly distilling the sulfide from solution. The sulfide is determined colorimetrically by conversion to methylene blue. Hydrogen sulfide concentrations of the order of 3 to 8 µg. may be estimated.

Numata has continued his studies of the determination of glutathione. By the use of a specific cysteine method (26), it was possible to develop techniques for the determination of glutathione in blood (27) and in tissues (28). The glutathione and cysteine concentrations of a number of tissues are given (28).

As a preliminary to the study of the oxidation of proteins by porphyrindin, Brand & Kassell (29) have established that cysteine and

glutathione are stoichiometrically oxidized by the dye to the disulfide stage at 0° and pH 7.2. The reaction is not affected by the presence of guanidine hydrochloride. During the course of a study of the partition coefficients of the N-acetyl derivatives of the naturally occurring amino acids between water and chloroform, Synge (30) reported a partition coefficient of 22 for acetyl-*l*-methionine and a value greater than one thousand for N,N'-diacetyl-*l*-cystine. This information was applied (31) to effect an 80 per cent recovery of methionine from a synthetic mixture of amino acids. This recovery was possible, however, because it could be based on total sulfur analyses, inasmuch as methionine was the only sulfur-containing amino acid present in the mixture.

Cysteine can be determined with the dropping mercury electrode in a medium of 0.1 N perchloric acid (17). Hydrochloric acid cannot be used since the anodic chloride wave interferes with the cysteine wave at this pH. The diffusion curve was found to be proportional to the concentration of cysteine. Homocystine and homocysteine can be determined quantitatively with the polarographic method (32). Concentrations as low as 26 μg. of amino acid per cc. have been estimated with an accuracy of ±3 per cent. It is not possible to distinguish between homocystine and cystine qualitatively by the polarographic method if these amino acids are present in solution together.

Sulfur and sulfur-containing amino acids in proteins.—Although a discussion of the state and behavior of sulfur in proteins under various conditions, e.g., denaturation, is presented elsewhere in this volume (p. 111), it appears appropriate to consider here certain observations concerned specifically with the concentration of sulfur and of sulfur-containing amino acids in proteins.

Further data are available (33) regarding the question of the presence of methionine in insulin. Hydrolysis of crystalline insulin with hydriodic acid resulted in the formation of 0.6 to 0.7 per cent volatile iodide calculated as methionine. The hydrolyzate, however, gave practically negative tests for homocysteine thiolactone, indicating that the volatile iodide which was obtained originated from some source other than methionine in the insulin molecule. Further proof that the volatile iodide did not arise from methionine was obtained from two types of experiments: (*a*) Preliminary hydrolysis of insulin with hydrochloric acid was found to result in a two-thirds decrease in the volatile iodide liberated by the insulin on digestion with hydriodic acid. There was no decomposition of methionine by treatment

with hydrochloric acid under these conditions in control experiments.
(b) It was not possible to identify the volatile alkyl iodide from me-
thionine as methyl iodide. The crystalline insulin used in this investi-
gation was prepared by crystallization from phosphate buffer in the
presence of zinc ion (34). However, the product was recrystallized
from a buffer system containing acetic acid, ammonia, and pyridine
(35). It is interesting to note that Sullivan & Hess (36) have reported
that the latter method of crystallization, while leaving the total sulfur
content unchanged, affects a part of the cystine present in the protein
in a manner so that loss of sulfur in a volatile form occurs during hy-
drochloric acid hydrolysis. Moreover, Sullivan & Hess found that
insulin crystallized by the zinc method did not show evidence of la-
bilized cystine during hydrolysis of the protein. Inasmuch as the
crystalline insulin employed by du Vigneaud, Miller & Rodden (33)
was recrystallized by a method which, according to Sullivan & Hess,
labilizes the cystine, it may appear that the volatile material found
in the study of the former authors had its origin to some extent
from the method of crystallization of the insulin. However, since
du Vigneaud, Miller & Rodden obtained similar data with amorphous
insulin, the method of crystallization used cannot explain their find-
ings. Moreover, the volatile product obtained on digestion with
hydriodic acid is apparently not methyl iodide, and it may be con-
cluded that methionine is not present in insulin. In view of their
implications and importance in protein chemistry, it is desirable to
have a repetition of the experiments of Sullivan & Hess (36).

Freudenberg & Münch (37) reported that the rate of hydrogen
sulfide liberation from insulin treated with $0.033 N$ sodium hydroxide
at 30° is slow and is not proportional to the inactivation of the hor-
mone. After removal of the hydrogen sulfide, sulfhydryl reactions are
still given by the inactive insulin. It is stated that if the pH is de-
creased to 10.5, insulin is inactivated in fifteen hours at 30° without
the liberation of ammonia or hydrogen sulfide, or the appearance of
sulfhydryl groups. Alkali-inactivated insulin was not regenerated in
the presence of cysteine, but insulin which had been inactivated by
cysteine was claimed to be regenerated by hydrogen peroxide.

Schöberl & Fischer (38) followed the changes in sulfur content of
various preparations of papain during the purification of the enzyme.
About half of the total sulfur was present as sulfate. Total sulfur con-
centration increased with purification; the rise was reflected particu-
larly in a marked increase in cystine content. Four different papain

preparations were analyzed and the following ranges of values found: total sulfur, 3.1 to 3.59 per cent; sulfate, 1.58 to 1.79 per cent; cystine sulfur, 0.57 to 1.58 per cent; and methionine sulfur, 0.24 to 0.32 per cent. All of the sulfur of native tobacco mosaic virus protein has been accounted for as 0.68 per cent cystine (39), and it therefore appears likely that this protein does not contain methionine. The sulfur in the denatured virus protein is present as cysteine. A rather high content of sulfur (1.9 per cent) has been found (40) in a hemolysin produced by Group A hemolytic streptococci of the β-type. Part of the sulfur exists as sulfhydryl groups, part as sulfate sulfur, and the remainder in undetermined forms. The activity of the hemolysin apparently is dependent upon the position of the disulfide-sulfhydryl equilibrium in the hemolysin molecule.

A study (41) of the chemical composition of nucleoprotein fractions from the livers of rabbits, rats, calves, and cows includes determinations of total sulfur, cystine-cysteine, and methionine. The sum of the cystine-cysteine and methionine accounts for practically all of the total sulfur in each product. The values are very nearly the same for all of the preparations, regardless of the species from which the nucleoprotein fractions were derived. Preparations of elementary bodies of vaccinia were found to contain 1.9 per cent cystine (42). The cystine content of the purified virus is considerably different from that of various materials discarded during the purification process. Highly purified seromucoid (from ox blood) on analysis contained 2.3 per cent cystine (43).

Routh (44) has continued his studies on powdered wool. A loss of cystine sulfur was observed during the prolonged grinding of wool in a steel mill. This loss was probably due to oxidation since more than 50 per cent of the water-soluble sulfur (after grinding) was in the form of inorganic sulfate. There was also evidence of the formation of intermediate oxidation products of cystine (cystine disulfoxide, sulfinic acid, and sulfenic acid). These data appear to provide further evidence that wool keratin is not homogeneous and that weakening of the mechanical structure is accompanied by oxidative changes and the formation of water-soluble fractions. The same laboratory has reported (45) an extensive investigation of the cystine, cysteine, and sulfur concentrations in 120 samples of human hair. There is a statistically significant greater concentration of cystine and cysteine in male hair as contrasted to female hair. Cysteine ranges for all hair examined were from 0.2 to 0.8 per cent; cystine values were 9.4 to

16.5 per cent, and sulfur values, 4.54 to 5.78 per cent. In contrast to earlier reports in the literature, red hair showed no striking diifferences from hair of other colors. There was no relationship between age and composition.

Protein fractions from human serum and from the urine of nephritic patients have been analyzed for sulfur and cystine contents (46). Serum globulin contained 3.43 per cent cystine, and serum albumin 4.75 per cent. The sulfur of total serum proteins amounted to 1.62 per cent and of this sulfur 4.32 per cent was present as cystine. For the urinary protein, 1.62 per cent sulfur was found with cystine values of 5.7 and 6.0 per cent for two samples obtained from the urine of a patient ingesting two different types of diets. Fractions obtained from pig serum by salting out procedures showed cystine variations from 2.75 to 5.39 per cent. Birkofer & Taurinš (47) found no differences in cystine and methionine contents of globins prepared from the blood of a large number of patients. Concentrations of the sulfur-containing amino acids of the globins prepared from two monkeys were the same as those observed in human globin. The values varied, however, from those obtained with both cow and horse globins, which in turn differed from one another. Still other data were found for the globins of the dog family. Several species of the latter, e.g., dog, fox, and jackal, had globins whose cystine and methionine contents were identical.

Studies of seleniferous proteins have also included observations on the sulfur in these proteins. In a preliminary communication (48), a crystalline selenium- and sulfur-containing substance isolated from the proteins of seleniferous grains has been assigned an empirical formula of $C_{21}H_{44}N_6Se_2SO_{12}$. The material contains α-amino nitrogen and has an equivalent weight of 130.5. The suggestion is made that the preparation is an isomorphous combination of two compounds, $C_7H_{14}N_2O_4Se$ and $C_7H_{14}N_2O_4S$, in the ratio of 2 to 1, respectively.

There was no parallelism between the distribution of cystine and that of selenium in fractions obtained from protein-free peptic digests of the liver of a rabbit fed selenized grain (49). The quantitative removal of selenium from seleniferous grain can be effected by reagents which, however, also produce a loss of the sulfur of the protein (50). The stability of selenium in proteins toward alkaline plumbite (51) is somewhat greater than that of sulfur under similar conditions. Procedures which increased the loss of sulfur generally augmented the rate of loss of selenium. The similarity that is observed in the behavior

of selenium and sulfur in seleniferous proteins supports the suggestion that selenium may take a place in the protein molecule analogous to sulfur. However, it should be pointed out that the isolated selenium-containing compound which has been described (48) contains selenium in an unusually stable form. It is, of course, possible that the selenium in a selenium-containing amino acid would be labilized by combination with other amino acids in peptide linkages. It is also possible that more than one form of organic selenium is present in selenium-containing proteins.

Metabolism.—Further experimental data have been obtained bearing on the interesting metabolic relationships between the sulfur-containing amino acids and choline. Cystine supplements accentuated the marked hemorrhagic enlargement of the kidneys resulting in young rats on a low choline diet (52). On the other hand, *dl*-methionine was effective in preventing the development of pathological changes. However, the ratio of methionine to cystine in the dietary protein was not the only factor determining the incidence of hemorrhagic kidney degeneration in rats. The toxicity of the diet increased as the casein level was raised to 25 per cent and then decreased as the casein concentration was further increased. The toxic action of cystine and the protective action of a high casein level were independent of the level of dietary fat (53).

The observation (54) that either choline or betaine would enable an animal to utilize homocystine or homocysteine in the diet in place of methionine has been further studied (55). The difference in the effectiveness of the two compounds (betaine is less effective than choline in supporting rats on a diet containing homocystine but free of cystine and methionine) is seen principally as a delay of several days in the response of growth following the administration of betaine to deficient animals. Injection of the dietary supplements yielded similar results. These data support the suggestion that betaine and choline furnish methyl groups to homocysteine for the formation of methionine (54). The functioning of the methyl group of methionine in biological methylation has now been clearly demonstrated in two laboratories. During the course of a study of the formation of creatine from glycocyamine by rat liver slices, Borsook & Dubnoff (56) found that of thirty-two amino acids and related substances tested, *dl*-methionine was the only compound which increased the quantity of creatine formed from glycocyamine. The increased creatine synthesis was approximately 50 per cent greater than in controls without added

methionine. The strong suggestion from these data that methionine may furnish the methyl group for the synthesis of creatine from glycocyamine is now conclusively established by a recent publication of du Vigneaud, Chandler, Cohn & Brown (4). Methionine was synthesized containing deuterium in the methyl group, i.e., trideuteriomethionine. The latter compound was fed at an average daily level of 70 mg. for three weeks to rats kept on a methionine- and choline-free diet. The choline chloroplatinates isolated from the tissues contained a surprisingly high proportion of deuterium. This is definite proof of the transfer of the methyl group of methionine to choline (transmethylation). Creatine was also isolated as creatinine from the muscle tissues of rats fed trideuteriomethionine. The isolated creatinine showed a deuterium concentration which establishes clearly the role of the methyl group of methionine in the synthesis of creatine. Although Borsook & Dubnoff demonstrated that choline, unlike methionine, cannot yield a methyl group directly to form creatine from guanidoacetic acid, du Vigneaud and his colleagues point out that choline does contribute a methyl group to homocysteine to form methionine. The latter, in turn, can methylate guanidoacetic acid. Therefore, since "homocysteine could act as an intermediary agent, transmethylation from choline to guanidoacetic acid might take place." This striking contribution greatly aids in establishing the nature of one relationship between the metabolism of choline and that of methionine. At the same time, demethylation of methionine is clearly demonstrated to be an important process in the metabolism of this amino acid. It will be of great interest to have more information regarding the transmethylation process. The identification of the product remaining after demethylation would clearly establish whether homocysteine is formed as the first stage of methionine catabolism. It is possible that transmethylation may be accompanied by simultaneous alterations in the demethylated, residual molecule, and the nature of these changes might throw light on the mechanism of the transformation of methionine to cystine. It is obvious that this problem is intimately connected with another of prime importance in sulfur metabolism, i.e., the nature and components of the system catalyzing transmethylation.

A relationship between the sulfur-containing amino acids and choline which is not fully elucidated is that concerned with the influence of supplementary cystine and methionine on the production of fatty livers in rats on a high fat diet. The mechanism of this influence has been sought with the aid of radioactive phosphorus (57). The effect

of cystine, cysteine, and methionine on the phospholipid turnover of the liver was determined in rats by feeding each amino acid by stomach tube at the same time the radioactive phosphorus (inorganic phosphate) was injected. Methionine (100 mg. per animal), produced a 26 per cent increase in the rate of phospholipid turnover in the liver as compared to controls receiving no methionine; 200 mg. doses of methionine produced a somewhat greater effect. The administration of 200 mg. of cystine or 200 mg. of cysteine hydrochloride also produced a similar increased phospholipid turnover rate in the animals. In a later study (58) as little as 35 mg. of methionine produced an augmented rate of phospholipid turnover. The stimulating effect of cysteine was again observed, while taurine and di(β-hydroxyethyl) sulfoxide were inactive. It is difficult to understand the similarity in the effect of cystine, cysteine, and methionine on phospholipid turnover in view of the established observation that whereas methionine has a definite lipotropic action, cystine and cysteine are nonlipotropic and, indeed, accentuate the extent of liver lipid deposition. That the influence on phospholipid turnover is not merely an amino acid stimulatory action is seen from the fact that seven other amino acids were tested and found to be inactive in this respect.

Further studies of the role of the sulfur-containing amino acids in the production of fatty livers have sought to determine whether the effects of various proteins depend on their content of cystine and methionine. Tucker, Treadwell & Eckstein (59) studied the lipotropic activity of a diet low in protein (5 per cent casein or edestin), and high in fat, supplemented with methionine and cystine to the extent that the sulfur-containing amino acid content of the ration was equivalent to that of a 20 per cent casein diet. The results were similar to those observed when the level of casein in the low protein diet was raised to 20 per cent. In other words, the data may be explained on the basis of the methionine and cystine content of the diet. Some disagreement with this conclusion is seen in the work of other investigators. Channon, Manifold & Platt (60), by the administration of 4 mg. of cystine daily, produced an increase of approximately 50 per cent over the already high level of lipids in the livers of rats on a diet low in casein and high in fat. Higher levels of cystine caused no further accentuation of liver lipid deposition. The action of cystine was effectively counteracted by 1.8 mg. of choline daily. The cystine effect was not observed when the dietary casein was replaced by egg albumin. Apparently the amount of cystine in albumin, even at the

relatively low levels fed, is sufficient to produce a maximum "cystine effect." In contrast with previous data from the same laboratory (61), indicating that no appreciable lipotropic action of methionine was demonstrable unless the amino acid supplement was raised to 0.5 per cent on a low casein diet, the present investigation (60) reports that the lipotropic action of methionine is observed when the added methionine is 0.2 per cent of the diet. Increases above this concentration have no augmenting action. The action of methionine (at its optimum) is about one fifth that of choline. The observed lipotropic effects of proteins could not be explained solely on the basis of their cystine and methionine contents. S-Methylcysteine and taurine did not influence liver lipid deposition. It was confirmed that homocystine behaves similarly to cystine in increasing the extent of liver lipid deposition (54, 62). Blood fat analyses showed no alterations occurring on cystine-supplemented diets. Best & Ridout (63) found that the isomers of methionine exert a lipotropic action which is essentially similar to that observed with the racemic mixture. Cystine and methionine added to the basal, fatty-liver-producing diet in amounts equivalent to that provided by a diet containing 30 per cent casein exerted an insignificant effect in comparison with a casein diet which provided the same quantities of these amino acids.

It would seem, therefore, that although the lipotropic action of methionine is clearly established (in contrast to the capacity of cystine to increase the extent of liver lipid deposition and the similarity in behavior of homocystine and cystine), the relative concentrations of cystine and methionine in proteins do not in all laboratories adequately explain the lipotropic action of these proteins. Inasmuch as investigators have differed in their choice of basal fatty-liver-producing diets, discrepancies among the data are difficult to explain fully. The diet employed by Eckstein and his colleagues (59) appears to be more highly purified in the sense that accurate knowledge is available regarding the composition of each constituent of this diet. Best & Ridout (63) have used a source of protein (meat powder) whose content of cystine and methionine is not definitely known. Channon, Manifold & Platt (60) administered 10 μg. of thiamin daily to each animal. It has already been demonstrated by McHenry (64) that the daily addition of 5 μg. of crystalline thiamin to a basal diet will cause a marked increase in the liver fat content of young rats. While it is of course true that all investigators have compared the results obtained with various supplements with those of a control group of animals

not receiving the supplements, there are data suggesting that the level at which various adjuncts to the diet are given may markedly alter the magnitude of the effectiveness of the supplement in producing either a lipotropic effect or in promoting liver fat deposition. It is obvious that although it is now definitely established that the methyl groups of methionine are involved in choline formation, another factor in the synthesis of choline, namely, the supply of aminoethanol, is also important. This fact may be concerned in determining the lipotropic effect of proteins.

The list of compounds which inhibit growth of rats on a low protein basal diet by creating a deficiency in the sulfur-containing amino acids, which are apparently used to detoxify the added foreign substance, has been extended to include methylcholanthrene, benzpyrene, pyrene (65), and diphenyl (66). In the study of polycyclic hydrocarbons (65), it was observed that varying amounts of each of these substances were required to produce similar growth inhibition. Dietary supplements of *l*-cystine, *dl*-methionine, and *l*-cystine disulfoxide produced a prompt stimulation of growth when added to the diets containing methylcholanthrene or benzpyrene. Other forms of sulfur (taurine, sodium sulfate) or of amino acid nitrogen (glycine) were ineffective in this respect. Injection of glutathione also produced an increased growth rate in animals ingesting the methylcholanthrene-containing basal diet. Similar data were obtained with the basal diet to which pyrene had been added, although in this instance only dietary supplements of *l*-cystine and *dl*-methionine were tested. The failure of growth resulting from the incorporation of diphenyl (66) in a low protein basal diet can be overcome by supplements of *l*-cystine or *dl*-methionine. Taurine and sodium sulfate were ineffective. The complete report of the influence of iodoacetic acid on sulfur metabolism, as seen in growth studies in the young rat, is now available (67). The superimposition of *l*-cystine, *l*-cysteine hydrochloride, *dl*-methionine, or *dl*-homocystine on a growth-inhibiting diet containing iodoacetic acid produced a prompt stimulation of growth with a resulting weight gain comparable to that observed in animals ingesting the basal diet alone. Subcutaneous injections of *l*-cysteine hydrochloride or glutathione were also capable of stimulating growth in animals stunted with iodoacetic acid. *d*-Cystine, taurine, sodium sulfate, *l*-phenyluraminocysteine, riboflavin, or riboflavin phosphoric acid were ineffective supplements. The large body of data which has been published demonstrating growth inhibition of rats by various compounds which may involve the sulfur-

containing amino acids for their conjugation and excretion strongly
suggests that these growth-inhibiting substances produce a specific de-
mand in the organism for the sulfur-containing amino acids—cystine
and methionine—for detoxication purposes. Either of the latter, or
compounds yielding cystine or methionine in metabolism, may stimulate
growth in the presence of these foreign toxic substances by fulfilling the
requirements for detoxication mechanisms and for the synthesis of
tissue protein.

Stekol (8) has isolated N-acetyl-S-benzylcysteine from the urine
of adult rats fed S-benzylglutathione. The data suggest that S-benzyl-
glutathione is hydrolyzed in the body to yield S-benzylcysteine, inas-
much as the latter has already been shown to be excreted as N-acetyl-
S-benzylcysteine when fed to rats (68). The same investigator has
recently reported (69) that the administration of dibenzyl disulfide to
rats results in the formation and excretion of hippuric acid in the
urine of these animals. These results aid in explaining the formation
of hippuric acid when S-benzyl-*d*-cysteine is ingested (70). It is sug-
gested by Stekol that benzylmercaptan or dibenzyl disulfide may be
formed *in vivo* from S-benzyl-*d*-cysteine via S-benzylthiopyruvic acid.
The instability of this thiopyruvic acid might lead to the formation of
either benzylmercaptan or dibenzyl disulfide. This work is also of in-
terest in view of the suggestion by Wood & Fieser (1) that mercaptan
and cysteine derivatives of certain carcinogenic hydrocarbons may
eventually undergo conversion to the mercapturic acid, possibly even
through the intermediary formation of the disulfide. As Stekol points
out, however, it appears clear that the metabolic formation of an
S-cysteine derivative does not necessarily mean an ultimate obligatory
formation of the corresponding mercapturic acid in metabolism. In-
deed, according to the data of Stekol it would appear more likely that
the corresponding hydroxy or carboxyl derivative would arise from
decomposition of the mercaptan or the disulfide, with the resulting ex-
cretion of, respectively, either a conjugated sulfuric acid ester or a
substance conjugated with glycine.

Further studies of enzyme systems acting on cystine and cysteine
have appeared. Desnuelle and his colleagues have reinvestigated
some of their published data[1] and have withdrawn all previous results
and conclusions regarding the anaerobic degradation of cysteine and
cystine by *B. coli*. In the newer studies, it has been demonstrated (71)

[1] *Ann. Rev. Biochem.,* **9,** 199 (1940).

that in *B. coli,* grown anaerobically in Liebig's broth, there is no enzyme capable of decomposing cysteine. When cysteine is added to the broth, however, the bacteria elaborate an enzyme ("cysteinase") which splits off equimolecular amounts of hydrogen sulfide and ammonia from cysteine, the optimum pH being 6.4. The action of this enzyme on *l*-cystine has also been determined (72). This process occurs in two stages: (*a*) the *l*-cystine is reduced by the bacteria to *l*-cysteine and (*b*), the latter is then desulfurized and deaminated by cysteinase. A study of the optical specificity of cysteinase (73) reveals that only the naturally occurring isomers of cysteine and cystine are attacked. This specificity is absolute and permits the isolation of pure $d(+)$cystine after the action of the bacteria on racemic cystine. Adaptation of this information to the development of a specific method for the quantitative determination of *l*-cysteine would appear feasible.

Desnuelle and his colleagues have extended these studies to *Propionibacterium pentosaceum* (74). Interesting differences are seen when comparisons are made with the data obtained with *B. coli*. The production of ammonia from cysteine by *P. pentosaceum* is not parallel to the formation of hydrogen sulfide but depends on the metabolites in the presence of which desulfurization takes place. In the presence of glucose practically no ammonia is formed. Moreover, *d*- and *l*-cysteine behaved identically. With cystine, however, the course of the reaction is quite different. The desulfurization of *l*-cystine requires the presence of a hydrogen donator, glucose or glycerol being used. Absolute optical specificity is exhibited when *d*- and *l*-cystine are studied separately; no hydrogen sulfide is released from *d*-cystine. This is an interesting variation in behavior of the bacteria toward the isomers of cystine as contrasted to those of cysteine. The mechanism of the release of hydrogen sulfide from cystine and cysteine by *P. pentosaceum* is evidently quite different from that used by *B. coli.*

To the enzyme systems in mammalian liver which have already been described[2] as being concerned with the metabolism of cystine and cysteine, Fromageot, Wookey & Chaix (75) have added an enzyme which they name desulfurase. The latter is present in dog liver, and anaerobically liberates hydrogen sulfide but not ammonia from *l*-cysteine. The enzyme thus differs from *l*-cysteinase which has been studied in the same laboratory (71, 72, 73). Under the experimental conditions employed, desulfurase had a maximum activity at 50° and

[2] *Ann. Rev. Biochem.,* **9,** 199 (1940)

pH 7. Desulfurase did not act on *d*-cysteine. The relative concentrations of this enzyme, as indicated by comparative activity measurements based on the production of hydrogen sulfide from *l*-cysteine, and compared to liver tissue (as 100), are as follows: liver 100, pancreas 10 to 30, kidney 7, muscle 1 to 2, and brain 0.3 to 0.7. It would be of considerable importance to know the nature of the other product or products formed by the action of desulfurase on *l*-cysteine.

During the course of a recent, careful study of transamination with a purified enzyme preparation, Cohen (76) has examined the extent to which a large number of individual amino acids are transaminated. In confirming an earlier conclusion (77) that the reaction is not a general one in which many amino acids are involved, but that it is limited with respect to substrate specificity, Cohen found no transamination with *l*-cysteine, *dl*-methionine, or glutathione. The observation that *l*-cysteic acid is transaminated to a small extent, reacting with both α-ketoglutaric acid and oxalacetic acid, is probably due to the fact that cysteic acid is a dibasic amino acid. Whether this finding is of metabolic significance is not apparent at the present time.

The role of the sulfur-containing amino acids in meeting the body requirements for protein synthesis has been examined specifically with respect to the synthesis of blood proteins. The administration of either *l*-cystine or *dl*-methionine in one gram doses daily to experimentally anemic dogs stimulated hemoglobin production (78); cystine was more effective than methionine in this respect. Dogs depleted of serum protein by hemorrhage and a low protein diet show (79) a regeneration of albumin after the daily administration of one gram of *dl*-methionine with the basal diet for seven days. The methionine supplement stimulated the production of almost as much serum albumin as did the administration of casein.

Burroughs, Burroughs & Mitchell (80) have observed that the withdrawal of methionine from a diet in which the nitrogen was supplied by a complete mixture of pure amino acids resulted in a progressive increase in negative nitrogen balance. The deficiency in methionine could not be compensated for by the addition of cystine. On the other hand, a negative nitrogen balance brought about by the withdrawal of cystine from the diet could be corrected by an addition of methionine. It would appear, therefore, that for the replacement of endogenous nitrogen losses in the rat, as is true for growth requirements, cystine can cover only the cystine demands, while dietary methionine can fulfill the need for cystine as well as methionine.

dl-Methionine sulfoxide is as effective as *dl*-methionine in promoting the growth of rats on a methionine-deficient diet (81). The effects of cystine and methionine in stimulating lactation in rats is believed (82) to be related to the capacity of these amino acids to make sulfur-deficient proteins nutritionally adequate rather than to an action as unique lactation stimulants.

Sandberg, Perla & Holly (83) have reported a steady rise in the excretion of neutral sulfur in the urine of rats following thymectomy. An increase of from 25 per cent of the total urinary sulfur before thymectomy to an average of 40 per cent following the operation is seen in the neutral sulfur of the urine. Excretion of inorganic and ethereal sulfate, expressed in percentage of total urinary sulfur, remains unchanged. These data seem worthy of further investigation inasmuch as few procedures are known by which the excretion of neutral sulfur in the urine may be augmented without the administration of a foreign, organic compound. The nature of the substance or substances in the urine responsible for the rise in neutral sulfur following thymectomy would be of considerable interest.

Basinski & Lewis (84) were unable to find a relationship between sulfur metabolism and indoluria. Twenty-two patients with indoluria had a normal cystine content of their fingernail clippings. It is believed therefore unlikely that indoluria is due to a failure of conjugation because of the lack of available sulfur. Cysteine hydrochloride injections had no effect on the regression of carcinoma in Line A albino mice (85).

The rather widespread use of colloidal sulfur as a therapeutic drug adds importance to a study of the absorption and oxidation of colloidal sulfur (86). The administered dose of colloidal sulfur in humans was quantitatively recovered in the urine as sulfate indicating complete absorption, oxidation, and rapid excretion. However, the report that sulfur administration will increase sugar tolerance in the normal and diabetic individual is not supported (87). Administration of colloidal sulfur to depancreatized dogs neither aggravated nor ameliorated the diabetes.

The folklore regarding disturbed sulfur metabolism in arthritis and the therapeutic use of sulfur preparations of various types with "marked improvement" have been subjected to critical study (88). No evidence was found of a sulfur deficiency or abnormality of sulfur metabolism in arthritic patients. The data reveal no indication of a need for, or benefit from, sulfur medication in the treatment of arthri-

tis. These findings are in agreement with the report (89) that the cystine content of the fingernails in arthritis has no specific diagnostic value.

An interesting study of cystinuria has been reported by Andrews & Andrews (90) who had the opportunity to again examine a cystinuric boy who had been studied (91) three years previously during pre-adolescence. At that time this boy showed very little increase in cystine excretion after methionine feeding. However, at the age of sixteen, a considerable cystine increase (30 to 35 per cent) was evident after doses of methionine up to 5 gm. per day. A diuresis produced by ascorbic acid feeding also induced an increased cystine excretion. In fact, this rise (up to 69 per cent) was greater than that which resulted from methionine administration. With controlled water intake following ascorbic acid feeding, the maximum increase in cystine excretion was only 16 per cent above the control period. Brand and his colleagues have continued their studies of canine cystinuria. Further investigations have been made (92) on the occurrence of cystinuria in a family of Irish terriers. Cystine excretion and sulfur distribution in the urine of normal and cystinuric members of this family of dogs are presented. To two breeds of dogs (dachshund and Irish terrier) has been added (93) a third breed (Scotch terrier) in which cystinuria has been found.

Sulfur-selenium metabolic relationships.—Although the metabolic fate of foreign substances in the animal organism is being considered in detail elsewhere in this volume (p. 265), the effect of selenium on sulfur metabolism and the relation of selenium to the sulfur-containing amino acids is of particular interest here. In view of the possibility that selenized proteins contain a selenium analogue of cystine, it is welcome to have information regarding the toxicity of this compound (94). The optically inactive, synthetic compound (called selenium-cystine) has a toxicity comparable to that of sodium selenite and sodium selenate and a much higher toxicity than that of other selenium-containing organic compounds which have been tested. The toxicity of selenium-cystine is approximately seven times that of the nitrogen-free analogue, i.e., β,β'-diselenodipropionic acid. Data supporting the conclusion that glutathione protects rats against death from a minimum fatal dose of selenium (as sodium selenite) have been obtained in the same laboratory (95). This finding is of interest in view of other data relating selenium to sulfur metabolism. A suggestion of the formation of a selenium-containing mercapturic acid is seen in the

recent demonstration (96) that the rate of selenium excretion in steers is greatly increased by the administration of bromobenzene.

Factors concerned with rendering administered selenium innocuous have been studied in several laboratories. Schultz & Lewis (97) demonstrated that 17 to 52 per cent of injected selenium (sodium selenite) was excreted by rats as a volatile compound through the lungs within eight hours after administration. Although the nature of the volatile selenium compound was not established, previous suggestions of the formation of dimethylselenide after selenium administration prompted the testing of methionine and choline chloride as potential sources of methyl groups. No influence was observed on the concentration of volatile selenium expired.

Several laboratories have examined the relationship between dietary protein and the toxicity of selenium. Lewis, Schultz & Gortner (98) reported that increasing the casein level of a basal diet from 6 to 30 per cent resulted in a significant decrease in the toxicity of sodium selenite for the young rat. Cystine added as a supplement to the low casein diet did not materially influence the toxic action of selenium. Supplementary methionine, however, under the same conditions produced an improved growth and greatly delayed the lethal action of selenium. Arachin, at a 15 per cent level, afforded no protection against similar quantities of selenium, but the addition of supplementary methionine to this protein greatly delayed manifestation of selenium toxicity. In view of the low methionine content of arachin, it would seem at first glance that methionine has a specific ability to attenuate the toxic effects of selenium. However, the authors point out that methionine may not be the sole factor concerned, inasmuch as when 6 per cent casein was supplemented with sufficient methionine to give a total methionine content of the diet approximately equal to that of a 30 per cent casein diet, the protective action against selenium, while striking, was not usually as great as when 30 per cent casein diets were fed. Smith & Stohlman (99) did not observe a protective action of methionine in rats ingesting selenium-containing diets. These investigators have confirmed the observation that the level of casein in the diet is a determining factor in regulating the severity of selenium toxicity, and have also studied the protective action of other proteins. It was found that the beneficial effects of casein could be simulated by several proteins, i.e., lactalbumin, ovalbumin, gelatin, the proteins derived from wheat, dried brewers' yeast, and desiccated liver. Gortner (100) has also determined whether pro-

teins other than casein afford protection against selenium administration. Lactalbumin was beneficial, although it did not give as consistently good results as casein. Edestin and gelatin failed to promote growth and offset the toxicity of the selenium.

It is obvious from the above data that further investigation is required to establish clearly the relation of methionine to selenium toxicity. Factors other than methionine appear to be concerned. It has already been pointed out that supplements of casein are much more effective in offsetting selenium toxicity than are dietary adjuncts of methionine, even though the latter amino acid is given in a concentration comparable to that accounted for by the increased level of casein in the diet. Moreover, on the basis of its methionine content, edestin should be equally as good as lactalbumin in promoting the survival of animals ingesting selenium. A single amino acid deficiency apparently does not explain the data fully, and other amino acids may be involved. It should further be kept in mind that differences exist among proteins with respect to their nutritive value. Certain discrepancies in the data of various laboratories may arise from the fact that investigators have differed in their choice of a source of selenium. The feeding of selenium at a level of thirty-five parts per million as sodium selenite (100) may produce a more acute toxicity than ten parts per million as wheat selenium (99). Under the former circumstances, quantitative differences in the capacity of proteins to prevent selenium toxicity may appear. It must also not be overlooked that the offsetting of selenium toxicity may not be directly connected with the dietary protein. The latter may exert an influence on metabolic processes which in turn are affected by selenium, e.g., certain enzymatic reactions. Overemphasis of the theoretically possible relationships between selenium and sulfur appears inadvisable on the basis of present knowledge.

Glutathione.—It is indeed unfortunate that a large number of papers concerned with glutathione have not been available to the reviewer, and that the space available permits the consideration of only a few of those which were examined.

Glutathione has been isolated from thymus tissue (101). The glutathione concentration in the blood of infants at birth was 54 mg. per cent (102). This value gradually decreased until the end of the third month of life, when a concentration below the adult level was reached and maintained through the eleventh year. Arterial blood contained less reduced glutathione than venous blood (27), although the total glutathione concentration is the same for both types of blood.

Total glutathione concentration of the blood, liver, lungs, and heart tissue of guinea pigs was increased to more than 50 per cent above normal in guinea pigs given a lethal dose of strychnine (103). Marked increases in the reduced glutathione content of blood and various tissues were found (104) in rats which had been forced to run daily in order to produce a condition simulating rigorous physical training. It would seem that the production of marked muscular activity by widely different methods apparently led to these increases in blood glutathione. It would be of interest to confirm these data and to ascertain whether similar results could be obtained as a result of muscular activity induced by other procedures.

The rate of turnover of glutathione in the body has been followed (105) by the administration of glycine containing nitrogen isotope to rats. Two hours later, 7.6 per cent of the nitrogen of the liver glutathione was derived from the administered glycine. A significant turnover of glutathione was also found in the intestine. The maximum rate of glutathione turnover in the liver is calculated as 22.3 per cent (intestine 9.6 per cent) in two hours. By contrast, in the same period only 1.6 per cent (intestine 1.5 per cent) of the protein glycine of the liver was exchanged. This experiment establishes the fact that glutathione is a very unstable and reactive compound in the living organism. The rate of turnover of glutathione was also compared with the rate of synthesis of hippuric acid. This was done by injecting subcutaneously in rats benzoic acid together with glycine containing heavy nitrogen. After five hours glutathione was isolated from the liver. The minimum rate of turnover of the tripeptide in the liver was calculated to be 23 per cent; the glycine in the liver glutathione contained less N^{15} than did the excreted hippuric acid. This experiment therefore offers no support for the hypothesis that glutathione furnishes glycine for hippuric acid formation.

Relationships of endocrine glands to glutathione metabolism have been studied in two laboratories. Interesting claims have been made (106) for the role of adrenal hormones, the latter apparently exerting a regulatory effect upon the concentration of glutathione in muscle tissue. Epinephrine administration to experimental animals is reported to produce an increase, and cortical extract a decrease, in glutathione concentration in muscle. These data, however, cannot be accepted without reservation inasmuch as the effects of the hormones were not demonstrable in normal animals but were manifested only in cats in which the adrenals had been removed. Moreover, differences

in glutathione concentration in the normal as contrasted to the adre-
nalectomized cats were not very striking. A role has also been claimed
for the thymus gland in glutathione metabolism. Following thymec-
tomy in rats (83) striking changes occurred in the relationship between
reduced and oxidized glutathione of the blood. The most interesting
alteration was encountered in oxidized glutathione concentration; this
rose to 60 per cent above normal two weeks after thymectomy, al-
though the change in total glutathione was slight. A peak value of
120 per cent of the original value for oxidized glutathione concentra-
tion was seen four weeks after operation. A slow decline then was
evident, but a month following thymectomy the rise still amounted to
80 per cent above normal. A return to the normal blood glutathione
picture was not seen until eighteen weeks after operation. In view of
the striking alterations in concentration of oxidized glutathione in the
blood following thymectomy it might be possible to adapt this change
to a method of assay which would perhaps permit examination of
thymus extracts for possible substances which induce these alterations
in blood glutathione. The injection of such extracts into both normal
and thymectomized animals should be of interest.

It has been reported (107) that there is a decrease in the gluta-
thione content of the liver of rats injected with dibenzanthracene as
compared to control animals. The data are, however, not very con-
vincing inasmuch as only averages and ranges of values are presented.
The variations are rather considerable and individual determinations
are not given.

Further definite information regarding the physiological functioning
of glutathione is to be greatly desired. With the availability of more
specific methods for the determination of this tripeptide, it is of im-
portance to have well-controlled experiments in this direction with an
examination of the many functions and relationships claimed for glu-
tathione in physiological processes.

LITERATURE CITED

1. WOOD, J. L., AND FIESER, L. F., *J. Am. Chem. Soc.*, **62**, 2674 (1940)
2. WOOD, J. L., AND VIGNEAUD, V. DU, *J. Biol. Chem.*, **131**, 267 (1939)
3. SCHÖBERL, A., AND BRAUN, H., *Ann.*, **542**, 274 (1939)
4. VIGNEAUD, V. DU, CHANDLER, J. P., COHN, M., AND BROWN, G. B., *J. Biol. Chem.*, **134**, 787 (1940)
5. VIGNEAUD, V. DU, DYER, H. M., AND HARMON, J., *J. Biol. Chem.*, **101**, 719 (1933)
6. TOENNIES, G., *J. Biol. Chem.*, **132**, 455 (1940)
7. CLARKE, H. T., *Org. Syntheses*, **20**, 23 (1940)
8. STEKOL, J. A., *Proc. Soc. Exptl. Biol. Med.*, **43**, 108 (1940)
9. MILLS, JR., E. J., AND BOGERT, M. T., *J. Am. Chem. Soc.*, **62**, 1173 (1940)
10. GULLAND, J. M., PARTRIDGE, M. W., AND RANDALL, S. S., *J. Chem. Soc.*, 419 (1940)
11. ANSON, M. L., *J. Gen. Physiol.*, **23**, 247 (1939–40)
12. ANSON, M. L., *J. Gen. Physiol.*, **23**, 321 (1939–40)
13. BERNHEIM, F., AND BERNHEIM, M. L. C., *J. Biol. Chem.*, **134**, 457 (1940)
14. DOHERTY, D. G., STEIN, W. H., AND BERGMANN, M., *J. Biol. Chem.*, **135**, 487 (1940)
15. REDEMANN, C. E., AND NIEMANN, C., *J. Am. Chem. Soc.*, **62**, 590 (1940)
16. BAPTIST, N. G., AND ROBSON, W., *Biochem. J.*, **34**, 221 (1940)
17. KOLTHOFF, I. M., AND BARNUM, C., *J. Am. Chem. Soc.*, **62**, 3061 (1940)
18. RUZICKA, L., GOLDBERG, M. W., AND MEISTER, H., *Helv. Chim. Acta*, **23**, 559 (1940)
19. PFIFFNER, J. J., AND NORTH, H. B., *J. Biol. Chem.*, **134**, 781 (1940)
20. REICHSTEIN, T., *Helv. Chim. Acta*, **19**, 29 (1936); REICHSTEIN, T., AND GOLDSCHMIDT, A., *Helv. Chim. Acta*, **19**, 401 (1936)
21. KOSCHARA, W., *Z. physiol. Chem.*, **263**, 78 (1940)
22. HALLETT, L. T., AND KUIPERS, J. W., *Ind. Eng. Chem., Anal. Ed.*, **12**, 360 (1940)
23. MARENZI, A. D., AND BANFI, R. F., *Biochem. J.*, **33**, 1879 (1939)
24. MEDES, G., AND STAVERS, E., *J. Lab. Clin. Med.*, **25**, 624 (1940)
25. FREUDENBERG, K., AND MÜNCH, A., *Z. physiol. Chem.*, **263**, 13 (1940)
26. FUJITA, A., AND NUMATA, I., *Biochem. Z.*, **300**, 264 (1938–39)
27. NUMATA, I., *Biochem. Z.*, **304**, 404 (1940)
28. NUMATA, I., *J. Biochem. (Japan)*, **31**, 35 (1940)
29. BRAND, E., AND KASSELL, B., *J. Biol. Chem.*, **133**, 437 (1940)
30. SYNGE, R. L. M., *Biochem. J.*, **33**, 1913 (1939)
31. SYNGE, R. L. M., *Biochem. J.*, **33**, 1918 (1939)
32. STERN, A., AND BEACH, E. F., *Proc. Soc. Exptl. Biol. Med.*, **43**, 104 (1940)
33. VIGNEAUD, V. DU, MILLER, G. L., AND RODDEN, C. J., *J. Biol. Chem.*, **131**, 631 (1939)
34. SCOTT, D. A., *Biochem. J.*, **28**, 1592 (1934)
35. VIGNEAUD, V. DU, JENSEN, H., AND WINTERSTEINER, O., *J. Pharmacol.*, **32**, 367 (1927–28)
36. SULLIVAN, M. X., AND HESS, W. C., *J. Biol. Chem.*, **130**, 745 (1939)

37. FREUDENBERG, K., AND MÜNCH, A., *Z. physiol. Chem.*, **263**, 1 (1940)
38. SCHÖBERL, A., AND FISCHER, M., *Biochem. Z.*, **302**, 310 (1939)
39. ROSS, A. F., *J. Biol. Chem.*, **136**, 119 (1940)
40. SMYTHE, C. V., AND HARRIS, T. N., *J. Immunol.*, **38**, 283 (1940)
41. GREENSTEIN, J. P., AND JENRETTE, W. V., *J. Natl. Cancer Inst.*, **1**, 91 (1940)
42. HOAGLAND, C. L., SMADEL, J. E., AND RIVERS, T. M., *J. Exptl. Med.*, **71**, 737 (1940)
43. RIMINGTON, C., *Biochem. J.*, **34**, 931 (1940)
44. ROUTH, J. I., *J. Biol. Chem.*, **135**, 175 (1940)
45. CLAY, R. C., COOK, K., AND ROUTH, J. I., *J. Am. Chem. Soc.*, **62**, 2709 (1940)
46. MURRILL, W. A., BLOCK, W. D., AND NEWBURGH, L. H., *J. Biol. Chem.*, **133**, 521 (1940)
47. BIRKOFER, L., AND TAURINŠ, A., *Z. physiol. Chem.*, **265**, 94 (1940)
48. HORN, M. J., AND JONES, D. B., *J. Am. Chem. Soc.*, **62**, 234 (1940)
49. WESTFALL, B. B., AND SMITH, M. I., *U.S. Pub. Health Repts.*, **55**, 1575 (1940)
50. WESTFALL, B. B., AND SMITH, M. I., *Cereal Chem.*, **16**, 231 (1939)
51. PAINTER, E. P., AND FRANKE, K. W., *J. Biol. Chem.*, **134**, 557 (1940)
52. GRIFFITH, W. H., AND WADE, N. J., *J. Biol. Chem.*, **132**, 627 (1940)
53. GRIFFITH, W. H., *J. Biol. Chem.*, **132**, 639 (1940)
54. VIGNEAUD, V. DU, CHANDLER, J. P., MOYER, A. W., AND KEPPEL, D. M., *J. Biol. Chem.*, **131**, 57 (1939)
55. CHANDLER, J. P., AND VIGNEAUD, V. DU, *J. Biol. Chem.*, **135**, 223 (1940)
56. BORSOOK, H., AND DUBNOFF, J. W., *J. Biol. Chem.*, **132**, 559 (1940)
57. PERLMAN, I., STILLMAN, N., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **133**, 651 (1940)
58. PERLMAN, I., STILLMAN, N., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **135**, 359 (1940)
59. TUCKER, H. F., TREADWELL, C. R., AND ECKSTEIN, H. C., *J. Biol. Chem.*, **135**, 85 (1940)
60. CHANNON, H. J., MANIFOLD, M. C., AND PLATT, A. P., *Biochem. J.*, **34**, 866 (1940)
61. CHANNON, H. J., MANIFOLD, M. C., AND PLATT, A. P., *Biochem. J.*, **32**, 969 (1938)
62. SINGAL, S. A., AND ECKSTEIN, H. C., *Proc. Soc. Exptl. Biol. Med.*, **41**, 512 (1939)
63. BEST, C. H., AND RIDOUT, J. H., *J. Physiol.*, **97**, 489 (1939–40)
64. McHENRY, E. W., *J. Physiol.*, **89**, 287 (1937)
65. WHITE, J., AND WHITE, A., *J. Biol. Chem.*, **131**, 149 (1939)
66. WEST, H. D., *Proc. Soc. Exptl. Biol. Med.*, **43**, 373 (1940)
67. STEVENSON, E. S., AND WHITE, A., *J. Biol. Chem.*, **134**, 709 (1940)
68. STEKOL, J. A., *J. Biol. Chem.*, **124**, 129 (1938)
69. STEKOL, J. A., *Proc. Soc. Exptl. Biol. Med.*, **45**, 693 (1940)
70. STEKOL, J. A., *Proc. Am. Chem. Soc.* (100th meeting, Detroit, 1940)
71. DESNUELLE, P., AND FROMAGEOT, C., *Enzymologia*, **6**, 80 (1939)

72. DESNUELLE, P., *Enzymologia*, **6**, 242 (1939)
73. DESNUELLE, P., *Enzymologia*, **6**, 387 (1939)
74. DESNUELLE, P., WOOKEY, E., AND FROMAGEOT, C., *Enzymologia*, **8**, 225 (1940)
75. FROMAGEOT, C., WOOKEY, E., AND CHAIX, P., *Compt. rend.*, **209**, 1019 (1939)
76. COHEN, P. P., *J. Biol. Chem.*, **136**, 565 (1940)
77. COHEN, P. P., *Biochem. J.*, **33**, 1478 (1939)
78. WHIPPLE, G. H., AND ROBSCHEIT-ROBBINS, F. S., *J. Exptl. Med.*, **71**, 569 (1940)
79. KEMMERER, K. S., AND HEIL, G. P., *Proc. Soc. Exptl. Biol. Med.*, **44**, 122 (1940)
80. BURROUGHS, E. W., BURROUGHS, H. S., AND MITCHELL, H. H., *J. Nutrition*, **19**, 363 (1940)
81. BENNETT, M. A., *Biochem. J.*, **33**, 1794 (1939)
82. HAAG, J. R., AND WRIGHT, L. D., *J. Nutrition*, **19**, 563 (1940)
83. SANDBERG, M., PERLA, D., AND HOLLY, O. M., *Endocrinology*, **26**, 102 (1940)
84. BASINSKI, D. H., AND LEWIS, G. T., *Proc. Soc. Exptl. Biol. Med.*, **42**, 628 (1939)
85. JONES, E. E., *Proc. Soc. Exptl. Biol. Med.*, **42**, 403 (1939)
86. GREENGARD, H., AND WOOLLEY, J. R., *J. Biol. Chem.*, **132**, 83 (1939)
87. GREENGARD, H., AND BURT, G. J., *Proc. Soc. Exptl. Biol. Med.*, **42**, 253 (1939)
88. FREYBERG, R. H., BLOCK, W. D., AND FROMER, M. F., *J. Clin. Investigation*, **19**, 423 (1940)
89. SULLIVAN, M. X., *Am. J. Surg.*, **43**, 620 (1939)
90. ANDREWS, J. C., AND ANDREWS, K. C., *J. Elisha Mitchell Sci. Soc.*, **55**, 203 (1939)
91. ANDREWS, J. C., AND RANDALL, A., *J. Clin. Investigation*, **14**, 517 (1935)
92. BRAND, E., CAHILL, G. F., AND KASSELL, B., *J. Biol. Chem.*, **133**, 431 (1940)
93. BLAMEY, E. R., AND BRAND, E., *Proc. Soc. Exptl. Biol. Med.*, **42**, 747 (1939)
94. MOXON, A. L., *J. Am. Pharm. Assoc.*, **29**, 249 (1940)
95. DuBOIS, K. P., RHIAN, M., AND MOXON, A. L., *Proc. S. Dakota Acad. Sci.*, **19**, 71 (1939)
96. MOXON, A. L., SCHAEFER, A. E., LARDY, H. A., DuBOIS, K. P., AND OLSON, O. E., *J. Biol. Chem.*, **132**, 785 (1940)
97. SCHULTZ, J., AND LEWIS, H. B., *J. Biol. Chem.*, **133**, 199 (1940)
98. LEWIS, H. B., SCHULTZ, J., AND GORTNER, JR., R. A., *J. Pharmacol.*, **68**, 292 (1940)
99. SMITH, M. I., AND STOHLMAN, E. F., *J. Pharmacol.*, **70**, 270 (1940)
100. GORTNER, JR., R. A., *J. Nutrition*, **19**, 105 (1940)
101. SCHAFFER, N. K., AND ZIEGLER, W. M., *Proc. Soc. Exptl. Biol. Med.*, **42**, 93 (1939)
102. McNAMARA, H., AND SENN, M. J. E., *Am. J. Diseases Children*, **59**, 97 (1940)

103. Santavy, F., *Compt. rend. soc. biol.,* **133,** 169 (1940)
104. Nitzescu, I. I., and Suciu-Stan, M., *Compt. rend. soc. biol.,* **133,** 518 (1940)
105. Waelsch, H., and Rittenberg, D., *Science,* **90,** 423 (1939)
106. Iljin, V. S., *Bull. biol. méd. exptl. U.R.S.S.,* **8,** 154 (1939)
107. Goerner, A., and Goerner, M. M., *Am. J. Cancer,* **37,** 518 (1939)

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CARBOHYDRATE METABOLISM

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Enzymatic Synthesis of Polysaccharide

The enzyme phosphorylase which catalyzes the reversible reaction, glycogen (or starch) + inorganic phosphate ⇌ glucose-1-phosphate, has been shown to be widely distributed in nature. It was originally shown to be present in extracts of muscle, heart, liver, brain, and yeast and has now been found in extracts of retina (146) and in extracts of a variety of plant tissues (66, 67). There are indications that the enzymes from these various sources, though closely related, are not identical. The enzymes prepared from brain, heart, liver, and yeast synthesize a polysaccharide which gives a brown color with iodine and resembles glycogen in all respects. The enzyme extracted from muscle synthesizes a polysaccharide which gives a blue color with iodine and resembles starch. X-ray photographs of natural potato starch and of starch synthesized by the action of potato phosphorylase give very similar patterns (4). The polysaccharide synthesized by muscle phosphorylase also gives x-ray diffraction patterns which are almost identical with those of potato starch (5). These observations offer a new approach to the problem of the constitution of the glycogen and starch molecules.

Wheat, rice, corn, and potato starch pastes are equally well phosphorylated in dialyzed muscle extract (33). Amyloses prepared from starches by electrophoresis are phosphorylated only a third to a half as rapidly as the original starch. Amylopectin, when treated with α-amylase, loses its ability to be phosphorylated much more rapidly than when treated with β-amylase.

The kinetics of the reaction, when studied with plant (68) and animal (27) phosphorylases, show great similarity. The following observation appears to be of special significance. When glucose-1-phosphate is added to crude enzyme preparations, there is an initial lag in the liberation of inorganic phosphate and the formation of polysaccharide. With further purification the reaction toward the left disappears completely, while full activity is retained toward the right (26). The activity toward the left is restored when a small

151

amount of glycogen or starch (1 to 5 mg. per 100 cc.) is added. As pointed out by Cori & Cori (27) and Hanes (68), this observation makes unnecessary the suggestion put forward by Kiessling (1939)[1] that two enzymes are involved, one synthesizing polysaccharide and the other breaking it down.

An analysis of the activating effect of the added glycogen on the reaction proceeding to the left shows that one is dealing with a combination of many enzyme molecules with an activating glycogen molecule (27). Perhaps the activating glycogen molecule serves as some sort of a pattern and this may represent a general principle in the enzymatic synthesis of other compounds of high molecular weight. The induction phase observed with purified potato phosphorylase is stated by Hanes (68) to be abolished not only by addition of starch but also by the addition of maltose. Muscle phosphorylase is not activated by purified maltose.

The position of the equilibrium of this reaction is influenced by the hydrogen ion concentration, as shown by Hanes for plant phosphorylase and by Cori & Cori for animal phosphorylases (muscle, brain, liver). The ratio of total concentrations of inorganic phosphate to glucose-1-phosphate (which may be reached from either side) is 5.7 at pH 6 and 2.7 at pH 7.6. As calculated by Hanes (68), the ratio of the divalent ions, $(HPO_4)^=/(C_6H_{11}O_5 \cdot O \cdot PO_3)^=$, in contrast to the ratio of total concentrations, remains approximately constant at a value of 2.2 over this pH range which indicates that the equilibrium is determined by the divalent ions only.

The rate of activity (to the left) rises with addition of increasing amounts of glycogen and approaches a maximum at a glycogen concentration of 200 to 500 mg. per cent, but the glycogen concentration does not influence the position of the equilibrium. One possible explanation is that owing to its high molecular weight the molar concentration of glycogen is too low to cause an appreciable displacement of the equilibrium (27).

Under optimal conditions (high initial glycogen concentration, presence of reduced glutathione) the reaction is first order. If unit enzyme activity is defined as a velocity constant of 10^{-3}, one obtains a value of 1,000 units per mg. of purified muscle phosphorylase (27).

Addition of adenylic acid is necessary for the activity of phosphor-

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[1] Authors' names followed by the year in parenthesis refer to citations given in previous volumes of the *Annual Review of Biochemistry*. Full references to these citations appear as an addendum at the end of this chapter.

ylase obtained from mammalian tissues. The phosphorylase–adenylic acid complex is 50 per cent dissociated at a concentration of 3×10^{-5} mols adenylic acid per liter. Inosinic acid has a slight activity, particularly with crude phosphorylase solutions (145). Phosphorylase from plant sources (yeast, pea, potato) does not require adenylic acid addition. It is still too early to state whether adenylic acid is bound so closely to the plant enzyme protein that it is not removed by procedures which remove it from the animal enzyme or whether the plant enzyme is active without this coenzyme.

Glucose inhibits phosphorylase activity in either direction, α-glucose being much more inhibitory than β-glucose. By varying the relative concentrations of glucose and glucose-1-phosphate it was shown that this inhibition is competitive (27). Fructose, mannose, galactose, glucose-6-phosphate, and maltose had hardly any inhibitory effect in concentrations in which glucose was strongly inhibitory. Phlorhizin and phloretin also inhibit the activity of the enzyme in either direction. In contrast to the glucose inhibition, the phlorhizin inhibition cannot be counteracted by increasing the glucose-1-phosphate concentration, but is diminished by increasing the concentration of adenylic acid.

The enzymatic synthesis and breakdown of glycogen has been reviewed by Cori (28, 29).

Glucose Phosporylation and Oxidation

Glucose-1-phosphate, formed from glycogen plus inorganic phosphate by the action of phosphorylase, is converted by another enzyme, phosphoglucomutase, to glucose-6-phosphate. This enzyme was found to accompany phosphorylase in all animal tissues examined as well as in yeast, and has recently been reported to be present in tissues of higher plants (66). Glucose-6-phosphate is also formed directly by an enzymatic transfer of phosphate from adenosinetriphosphate to glucose and is therefore an intermediate common to the metabolic pathways of glycogen and of glucose. A phosphorylation of glucose on carbon atom 1 has not yet been demonstrated.

The aerobic phosphorylation of glucose in cell-free kidney extracts which was discovered by Kalckar (1937) has been investigated further by Colowick et al. (22). Glucose and inorganic phosphate disappeared and phosphorylated products accumulated if fluoride was added. The following were found to be essential components of the system as determined by reactivation of aged or of dialyzed extracts: (a) an oxidizable substrate (citrate, glutamate, α-ketoglutarate, succinate);

(b) adenylic acid and cozymase; (c) magnesium ions which are necessary for both the oxidation of the substrates mentioned and the transfer of phosphate to glucose. The product which accumulated in the presence of fluoride was a mixture of fructosediphosphate and phosphoglyceric acid with the former predominating. The oxidation of succinic to fumaric acid was shown to effect phosphorylation of glucose, and it was suggested that substrates, e.g., pyruvic acid, whose oxidation involves C_4-dicarboxylic acid catalysis might effect phosphorylation.

In addition to the substrates mentioned under (a), glucose and pyruvic acid were found to serve as oxidizable substrates, provided a small amount of fumaric acid was added to the dialyzed extracts (23). In the absence of fluoride, fumaric acid catalyzed the oxidation of both glucose and pyruvic acid and only small amounts of phosphate esters accumulated. In the presence of fluoride, fumaric acid no longer catalyzed the oxidation of glucose, though it still catalyzed the oxidation of pyruvic acid. Phosphate analyses showed that pyruvic acid oxidation, in the presence of glucose and fluoride, caused a marked phosphorylation of glucose with almost complete disappearance of the inorganic phosphate added. These experiments show that glucose in order to be oxidized by this system must first be transformed to pyruvic acid. This transformation involves an initial phosphorylation of the glucose molecule, followed by dephosphorylation of the phosphopyruvic acid formed. Fluoride inhibits dephosphorylation and thus prevents the formation of pyruvic acid, the oxidation of which is necessary for the initial reaction, i.e., the phosphorylation of glucose. Similar results were obtained with homogenized brain tissue.

Ochoa (112) reported that glucose and hexose-6-phosphate are phosphorylated to hexosediphosphate in brain dispersions when pyruvic acid is used as oxidizable substrate with fumarate as catalyst, and that adenylic acid acts as the phosphate transfer agent. He also showed that, when oxidation of pyruvic acid was inhibited by arsenite, phosphorylation of glucose could be brought about by the oxidation of succinate to fumarate, but that the ratio of phosphate esterified to oxygen consumed was twice as high with pyruvate as substrate as with succinate plus arsenite. From this he concluded that one half of the phosphate esterified during pyruvate oxidation is connected with the dehydrogenation of pyruvic acid and the other half with the hydrogen transfer by means of the dicarboxylic acid system. Belitzer & Tsiba-

kova (13) found in experiments with minced muscle, in which crea-
tine served as phosphate acceptor, a still higher ratio of phosphate
esterified to oxygen consumed (three atoms of phosphorus per one
atom of oxygen). This indicates that in addition to the primary dehy-
drogenation of the substrate, two subsequent hydrogen transfers may
cause phosphorylation. Belitzer & Golovskaya (12) found that hex-
osemonophosphate is an even better phosphate acceptor than creatine
but that glucose is not phosphorylated by muscle mince unless hexo-
kinase from yeast is added; this enzyme transfers phosphate from
adenosinetriphosphate to glucose. Hexokinase is either deficient or
more labile in muscle than in other tissues.

The aerobic phosphorylation of glucose and other phosphate accep-
tors is of importance because it is a mechanism by which oxidative
energy is utilized in the cell. The adenylic acid system serves as the
mediator of this energy transfer and therefore plays a role not only in
anaerobic reactions but also in respiration. Aerobic phosphorylation
is involved in the conversion of fructose, glycerol, pyruvate, and lac-
tate to glucose in liver slices. When kidney slices are incubated aero-
bically with pyruvate and fumarate, a synthesis of glucose takes place
which in the case of avitaminotic animals is markedly accelerated by
addition of diphosphothiamin (10). The synthesis may take place
by way of phosphopyruvic acid; this substance was formed when
malate was added to kidney extract under aerobic conditions [Kalckar
(1939)] and when lactate was added to cat muscle incubated aerobi-
cally in the presence of fluoride (46).

PYRUVATE OXIDATION

The oxidation of pyruvic acid may be assumed to represent the
main path of carbohydrate breakdown in nonglycolyzing cells. In
glycolyzing cells pyruvate competes with oxygen as hydrogen accep-
tor and is reduced to lactic acid. Although diphosphothiamin is neces-
sary for the oxidation of pyruvate, it has not been shown to undergo
reversible reduction and oxidation (143).

Banga et al. (7) found that finely ground and dialyzed prepara-
tions of pigeon brain needed the presence of the following substances
for the oxidation of pyruvate: diphosphothiamin, inorganic phosphate,
a C_4-dicarboxylic acid (in catalytic amounts), adenylic acid or adeno-
sinetriphosphate, magnesium ions, and probably cozymase. Citrate
and α-ketoglutarate, i.e., intermediates of the citric acid cycle, were
much less active than the C_4-dicarboxylic acids (succinate, fumarate,

etc.) in the catalysis of pyruvate oxidation; it seems therefore unlikely that the main path of pyruvate oxidation in brain is through the citric acid cycle. A mechanism involving phosphorylation and phosphate transfer by means of the adenylic acid system is suggested by the necessity of inorganic phosphate for pyruvate oxidation and by the effect of adenylic acid. The latter, when added in the presence of the other components of the system, not only increased the oxidative removal of pyruvate, but also made its oxidation more complete. It seems likely that adenylic acid serves here as phosphate acceptor for some phosphorylated intermediate which is formed during pyruvate oxidation. This phosphorylated intermediate is apparently not acetylphosphate. Lipmann (91) has shown that during oxidation of pyruvate with enzymes prepared from lactic acid bacteria a substance is formed whose properties closely resemble those of acetylphosphate. When bacterial enzymes were used, acetylphosphate was found to transfer its phosphate to adenylic acid. This transfer has not been observed, however, with an animal enzyme system [Ochoa, Peters & Stockton (1939)].

Krebs & Eggleston (86) have brought forward additional evidence for the existence of a citric acid cycle in pigeon breast muscle and in other tissues. According to this scheme pyruvic acid condenses with oxalacetic acid to form citric acid which is oxidized to oxalacetic acid; this completes the cycle which may be sketched as follows:

$$\text{Fumarate} \xrightarrow{+\text{O}} \text{oxalacetic} \xrightarrow{+\text{pyruvate}+\text{O}} \text{citrate} (+\text{CO}_2) \xrightarrow{+\text{O}} \alpha\text{-keto-}$$

$$\text{glutarate} (+\text{CO}_2) \xrightarrow{+\text{O}} \text{succinate} (+\text{CO}_2) \xrightarrow{+\text{O}} \text{fumarate.}$$

If all the hydrogen is transferred to oxygen, the over-all of these reactions is: (1)

$$\text{pyruvate} + 2\tfrac{1}{2}\,\text{O}_2 \rightarrow 3\,\text{CO}_2 + 2\,\text{H}_2\text{O}.$$
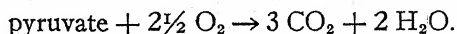
In the presence of large amounts of malonate the citric acid cycle is interrupted at the succinate stage due to inhibition of the succinic dehydrogenase. The reaction observed under these conditions conforms to the equation: (2)

$$\text{pyruvate} + \text{fumarate} + 2\,\text{O}_2 \rightarrow 3\,\text{CO}_2 + \text{H}_2\text{O} + \text{succinate.}$$
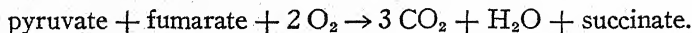
In the absence of malonate this is followed by the oxidation of succinate: (3)

$$\text{succinate} + \tfrac{1}{2}\,\text{O}_2 \rightarrow \text{fumarate} + \text{H}_2\text{O}.$$
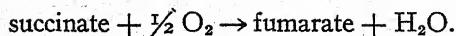
Thus the oxidation of pyruvate is completed. In interpreting the malonate experiments, it should be emphasized that in control experiments no anaerobic reduction of fumarate to succinate took place. In order to explain the stoichiometric relations shown in equation 2, an oxidative formation of succinate via citric acid is assumed which is not inhibited by malonate. In further confirmation of this scheme Krebs & Eggleston have shown that when an excess of pyruvate is added, citrate and α-ketoglutarate accumulate instead of succinate, the former in a yield of 15 and the latter of 50 per cent.

Baumann & Stare (11) found that the respiration of pigeon breast muscle, inhibited by malonate, was restored by the addition of fumarate, malate, or α-ketoglutarate, while citrate failed to restore respiration. From this they concluded that the entire citric acid cycle is not essential for the respiration of muscle, although parts of it may be essential. If citric acid is not an essential intermediate in the respiration of muscle, some other reaction mechanism which would explain equation 2 would have to be found. An inhibition of the respiration in dispersions of brain tissue by malonate in the presence of pyruvate or lactate was shown by Banga et al. (1939), while Huszák (77) obtained no inhibitory effect of malonate in brain brei. It has been shown by the former authors that substances which affect the metabolism of dispersions may have less effect on brei because they do not permeate rapidly into the cells.

In heart muscle the reactions comprising the citric acid cycle were found to take place (131). In pigeon liver (43), besides the citric acid cycle, two other reactions have been observed which cause a rapid removal of pyruvate. The first of these is the formation of acetoacetate from pyruvate which has been described before; the second is the formation of α-ketoglutarate which takes place when large amounts of pyruvate are added and which does not require the addition of C_4-dicarboxylic acid. Evans & Slotin (44) studied this reaction in minced liver in a medium containing bicarbonate prepared from radioactive carbon (C^{11}). Since the α-ketoglutarate which they isolated as the 2,4-dinitrophenylhydrazone was strongly radioactive, they postulate the reaction: pyruvate $+ CO_2 \rightarrow$ oxalacetate; the latter would then combine with another molecule of pyruvate to form α-ketoglutarate by way of citrate.

Wood & Werkman (155) have shown that propionic acid bacteria utilize carbon dioxide during fermentation of glycerol and that suc-

cinic acid is formed in amounts equivalent to the quantity of carbon dioxide absorbed. Wood *et al.* (156) using heavy carbon (C^{13}) and Carson & Ruben (21) using radioactive carbon (C^{11}) have demonstrated that the succinic acid formed during this fermentation contains the tracer carbon. The former authors have shown that it is present in the carboxyl groups. It is of great interest that a reaction in which carbon dioxide is utilized in the formation of a carbon to carbon bond (i.e., a reversal of decarboxylation) also occurs in animal tissues; it provides one of the mechanisms for the formation of the essential C_4-dicarboxylic acids.

Krebs (87) assumes that the reversible system, oxalacetate $\rightleftharpoons$ *l*-malate, acts as hydrogen carrier in three reactions: in the oxidation of "triose" to pyruvate; in the oxidative condensation of pyruvate plus oxalacetate to form citrate; and in the oxidation of citrate to α-ketoglutarate. The Szent-Györgyi cycle would therefore be concerned with the transport of (at least) one half of the hydrogen atoms released during the oxidation of one triose molecule. These are reactions in which pyridine nucleotides are interposed as hydrogen carriers, and while Krebs and others have shown that oxalacetate can act as hydrogen acceptor under anaerobic conditions, there is in the opinion of the reviewers no direct evidence available that oxalacetate $\rightleftharpoons$ *l*-malate acts as carrier under aerobic conditions. Furthermore, as pointed out by Ball (6) the reversible reaction, reduced cozymase + oxalacetate $\rightleftharpoons$ cozymase + malate, leaves the conditions the same as at the start with the reduced cozymase still to be oxidized.

As regards the reversible system, fumarate $\rightleftharpoons$ succinate, Potter (117) has shown that in an enzyme system consisting of triosephosphate and succinic dehydrogenase, flavoprotein and cytochrome—cytochrome oxidase, the oxygen consumption was unaffected by an amount of malonate which caused complete inhibition of succinic dehydrogenase. Krebs (87) also regards it as doubtful that this pair acts as a hydrogen-transporting system. Yet the observation that the oxidation of succinate to fumarate is coupled with phosphorylation emphasizes the fact that hydrogen transport by means of this pair may have some physiological significance.

Sober *et al.* (132) have shown that citrate excretion is diminished in thiamin-deficient rats and that the increase in citrate excretion due to succinate injection is much less than in normal animals. Administration of thiamin to thiamin-deficient rats caused in a few days a ten-fold increase in citrate excretion. The authors conclude that di-

phosphothiamin is essential for the synthesis of endogenous citric acid.

Delrue & Dekeyser (36) reported that the disappearance of intravenously injected pyruvate in rabbits was markedly accelerated by insulin injections. A drop in the normal level of blood pyruvate following insulin injections has been reported by Euler & Högberg (42). A review of the mechanism of hydrogen transport in animal tissues has been published by Potter (118) and the chemical reactions in which diphosphothiamin participates are discussed by Lipmann (91a), Peters (115a), and by Lipton & Elvehjem (92).

GLYCOLYSIS

Dische (39) confirmed MacFarlane's observation (1939) that inorganic phosphate disappears in fermenting living yeast cells. The peak of ester formation was reached one minute after addition of glucose to the yeast. The esterified phosphate was shown to be made up of hexose di- and monophosphate and of adenylpolyphosphate.

Holmes (76) found that sodium fluoride inhibited the disappearance of glucose in extracts or brei of tumor tissue and in extracts of retina. The assumption that one is dealing here with a new effect of fluoride, namely an inhibition of glucose phosphorylation, seems unnecessary. In a respiring system large amounts of glucose can be phosphorylated in the presence of sodium fluoride because adenosinetriphosphate, the phosphate donor for glucose, is constantly regenerated at the expense of oxidative energy. The fact that glucose did not disappear in the anaerobic experiments of Holmes is presumably due to a lack of regeneration of adenosinetriphosphate in the absence of dismutations.

Süllmann & Brückner (146) and Kerly & Bourne (83) studied the glycolytic mechanism in cell-free extracts of retina; the enzymatic reactions leading to the formation of lactic acid from glycogen were found to be the same as in muscle. Glucose was glycolyzed more rapidly than glycogen. Retinal extracts appear to be rich in the enzyme which transfers the labile phosphate of adenosinetriphosphate to glucose. This enzyme (hexokinase) must be added to muscle extracts in order to enable them to glycolyze glucose. Kobayasi (85) obtained an active hexokinase preparation from tumor tissue. The same author also showed that a dismutation between triosephosphate and pyruvic acid occurred in tumor extracts provided adenosinetriphosphate and cozymase were added. Geiger (50) prepared extracts of brain which

were active glycolytically; an inhibitory substance was present, the effect of which was nullified by dilution of the extract. Lactic acid was formed more rapidly from glucose, fructose, and mannose than from glycogen. Inorganic phosphate, adenosinetriphosphate, cozymase, and magnesium ions were found to be essential for glycolysis in brain and the usual phosphorylated intermediates (hexosediphosphate in the presence of iodoacetate, and phosphoglyceric acid in the presence of fluoride) were shown to be formed. Curiously enough, hexosemono-phosphate or hexosediphosphate, when added to brain extract, were not converted to lactic acid. Ochoa (113) repeated these experiments and confirmed the observation that dilution of the brain extract is necessary in order to obtain lactic acid formation; he found, however, that hexosemonophosphate and hexosediphosphate were glycolyzed as rapidly or more rapidly than glucose. (Creatine was added as phos-phate acceptor when the diphosphate was used.) The same enzymatic processes have therefore now been shown to occur in brain and in muscle. This is of some interest, since brain was considered for some time to be an example of a tissue with a nonphosphorylating gly-colysis.

Evidence for a number of reactions characteristic of phosphory-lating glycolysis has been obtained by Meyerhof & Perdigon (107) in extracts of retina, liver, kidney, and of rat and chick embryo. Needham et al. (108) maintain, however, that aged chick embryo brei forms lactic acid from glucose without addition of any coenzymes and in the absence of a demonstrable dismutation between triosephosphate and pyruvic acid. Meyerhof points out that the added phosphorylated compound may not have penetrated into the cells.

The experiments of Sacks (126) also fall under the heading of nonphosphorylating glycolysis. He found that when cats were injected with radioactive phosphate and muscle was removed two hours later, the labeled phosphate showed a differential distribution among the phosphorus compounds present in resting muscle. Thus the relative radioactivity per mg. of phosphate was 100 for inorganic phosphate, 7 to 16 for phosphocreatine, and 4 to 17 for the labile phosphate groups of adenosinetriphosphate. This differential distribution was retained when the muscle was tetanized for fifteen seconds and from this he concluded that the interchange of phosphate groups pos-tulated by the Embden-Meyerhof scheme does not take place in con-tracting muscle: that is, inorganic phosphate, phosphocreatine, and adenosinetriphosphate do not take part in the formation of lactic acid;

and hexosemonophosphate, which he assumed to be formed from phosphocreatine and glycogen, is not regarded as an intermediate in the formation of lactic acid.

The isolation of enzymes concerned with the reactions described in the Embden-Meyerhof scheme continues to be a fruitful field. Herbert et al. (74) isolated zymohexase, the enzyme which catalyzes the reversible reaction, hexosediphosphate $\rightleftharpoons$ 2 triosephosphate, as a homogeneous protein which is contained in the myogen fraction of muscle and constitutes about 1 per cent of the total muscle proteins. Straub (144) prepared crystalline lactic dehydrogenase which catalyzes the reversible reaction, lactate + cozymase $\rightleftharpoons$ pyruvate + reduced cozymase.

Human spermatozoa in Ringer's solution containing glucose show strong glycolysis and practically no respiration (100). Warren (150) measured respiration and aerobic and anaerobic glycolysis of bone marrow in serum and in Ringer's solution containing phosphate and glucose, and correlated these measurements with various cellular components in bone marrow. In serum, respiration and anaerobic glycolysis were 70 per cent higher than in the Ringer's medium. Lutwak-Mann (97) and Hills (75) studied the enzyme systems of articular cartilage. Glycolysis was not very active, partly because coenzymes were rapidly destroyed by the strong phosphatase present. A dismutation of triosephosphate and pyruvate was observed by both authors. Rudney (121) reported that l-glucose is not utilized by mammalian tissues and by bacteria. Agents which inhibit glycolysis have been reviewed by Gemmill (51).

INTESTINAL ABSORPTION

Phosphate analyses of the intestine have been made in order to test the validity of Verzár's theory of selective absorption of certain hexoses which are assumed to undergo phosphorylation and dephosphorylation in the intestinal mucosa. It was reported by Laszt & Süllmann (1935) and confirmed by Lundsgaard (1939), Eiler et al. (41) and Reiser (119) that a decrease in inorganic phosphate and increase in ester phosphate occur during absorption of glucose and fructose; the nature of the ester which accumulates has not been established. It should be pointed out that the accumulation of a phosphate ester in the mucosa might be connected with an increased rate of carbohydrate metabolism without being in any way connected with the absorption

mechanism. These observations neither support nor invalidate Verzár's hypothesis. More significant, perhaps, is the fact that fructose, glucose, and mannose are rapidly phosphorylated in various tissues, while galactose is very poorly phosphorylated; yet galactose is the most rapidly absorbed sugar, while mannose is absorbed at a very slow rate.

Kjerulf-Jensen & Lundsgaard (84) injected a large dose of sodium cyanide into rats which were absorbing fructose. Thereby further phosphorylations were inhibited and it was possible to measure the breakdown of the newly formed, easily hydrolyzable ester. It was found that the breakdown was rapid enough to comply with the assumption that the absorption of fructose involves its phosphorylation and dephosphorylation in the intestinal mucosa. The esters present in a nonabsorbing mucosa were split less rapidly after the cyanide injection. It would seem highly desirable to show that one is actually dealing with a fructose phosphate.

According to Verzár the slow rate of absorption of pentoses is explained by a process of simple diffusion, while the rapid absorption of "selective" hexoses is explained by processes of phosphorylation and diffusion. However, the pentose, xylose, is as rapidly absorbed as glucose from the ileum of cats (35). That configuration is an important factor influencing the rate of absorption of various sugars is clearly shown by the following data which are given in milligrams of sugar absorbed per 100 gm. rat per hour: l-xylose, 7; d-xylose, 74; l-xylulose, 44; d-xylulose, 131. The latter pentose was absorbed as rapidly as the "selective" hexose, fructose (88).

The decreased rate of glucose absorption in adrenalectomized rats (or in hypophysectomized rats in which the adrenal cortex is atrophic) is explained by Verzár (149) by the lack of an adrenal cortex hormone which is necessary for phosphorylations in general, i.e., of sugars, fats, and vitamins B_1 and B_2. For vitamins it has been clearly shown that their phosphorylation does not depend on an adrenal factor (47, 114, 122). Verzár's claim that riboflavin phosphate prolongs the life of adrenalectomized animals has also not been confirmed (18, 109). Since it seems doubtful that the adrenal cortex elaborates a factor necessary for phosphorylation, the slow rate of glucose absorption in adrenalectomized animals cannot be advanced as proof that the mechanism of absorption involves phosphorylation. Anderson & Herring (1) found that a high salt diet or injection of desoxycorticosterone improved the rate of absorption of glucose in adrenalectomized

rats. This would make it appear that the ionic imbalance of the tissues, or the loss of sodium and chloride ions, was responsible for the failure of glucose absorption. It seems more likely that it is the inanition of the adrenalectomized rats which is responsible for the slow rate of glucose absorption (116); fasting, even in normal animals, markedly reduced the rate of absorption; after a period of forced feeding the rate of absorption could be increased up to 100 per cent in adrenalectomized rats. Marazzi (101) attributes the effect that sodium chloride has on glucose absorption to the abolition of anorexia and undernutrition. She found that the rate of xylose absorption was not reduced in adrenalectomized animals.

GLYCOGEN DEPOSITION AND DISAPPEARANCE

Fenn & Haege (45) showed that for each gram of glycogen deposited in the liver of the cat an average of 1.63 ± 0.3 gm. of water is retained. (Recalculation to the same basis of older data obtained by Kaplan & Chaikoff [1936] showed that the dog retained an average of 1.46 ± 0.21 gm. of water.) Of the 1.63 gm. water, 0.45 gm. is accompanied by chloride ions and therefore extracellular. The remainder is intracellular and contains appropriate amounts of potassium ions, i.e., the concentration of potassium ion in the cell water is not diminished by an increase in glycogen content. The possible effect of a retention of potassium and phosphate ions in the liver on the concentration of these ions in the blood during glycogen deposition should be pointed out.

MacKay et al. (98) fed rats equivalent amounts (with respect to carbon) of glucose, glycine, or alanine. The highest glycogen content of the liver was reached after six hours with glucose, after fourteen hours with glycine, and after ten hours with alanine. The authors think that the remarkable glycogenetic power of these amino acids, as good or better than that of glucose, has been missed heretofore because glycogen deposition from them only starts several hours after feeding. Hall et al. (65) state that dl-threonine and dl-allothreonine form glycogen in the liver of rats. Newman et al. (111) noted glycogen deposition when they perfused cat livers with blood to which propylene glycol had been added. Other substituted glycols formed no glycogen.

Hastings et al. (72) fed 150 mg. of radioactive dl-lactate to rats. The lactate contained the C^{11} in the carboxyl group. The liver glycogen increased by 50 mg. in two and a half hours. [Only the l $(+)$-

lactate forms liver glycogen]. The radioactivity of the glycogen was only from 1 to 3.6 per cent of the administered lactate, while the expired carbon dioxide contained more than 10 per cent of the C^{11} administered as lactate. The authors suggest either that lactate is decarboxylated before it is converted to glycogen or that the glycogen is formed from another substance.

Bobbit & Deuel (17) determined the disappearance of glycogen from samples of liver which were incubated at 37° for different periods of time. In one hour only 31 per cent of the glycogen disappeared from rat liver, and only 10 to 16 per cent from rabbit, dog, or guinea pig liver. The authors point out that the disappearance of glycogen from the excised liver is less rapid than is generally supposed and that considerable glycogen is still present after many hours. It should not be overlooked, however, that the most rapid glycogen breakdown occurs in the first few minutes, as can be shown by the fact that unless special precautions are taken, the fermentable sugar content of the liver immediately after removal is much higher than the blood sugar.

Gemmill (53) stimulated the gastrocnemius in phlorhizinized rats. No significant decrease in fat content was noted. The carbohydrate content decreased in every case; in muscles with low glycogen stores (0.1 to 0.2 per cent) only 0.04 per cent disappeared. In this connection it is interesting that Winter & Knowlton (154) found normal tension development when the gastrocnemius of fasted rats was stimulated. Flock & Bollman (48) investigated resynthesis of glycogen in previously stimulated muscle. The rate of resynthesis was increased by injection of glucose but not by injection of lactate or insulin. Epinephrine did not prevent resynthesis.

Willstätter & Rhodewald (153) found that when muscle *brei* was incubated with a yeast extract (containing the enzyme hexokinase) less glycogen disappeared in the presence than in the absence of added glucose, from which they concluded that the latter was converted to glycogen. Similar results were obtained by Iri (80) who added glucose and hexokinase to muscle extract. Willstätter & Rhodewald believe that under physiological conditions glucose must first be converted to glycogen before it can be split to lactic acid in muscle and fermented to alcohol and carbon dioxide in yeast.

Goda (55a), Kruyk & Klingmüller (1939), and Mirski & Wertheimer (1939) have investigated the question as to whether or not glycogen deposition occurs during the induction period of yeast fer-

mentation. The first investigator found it to occur only in fresh but not in aged yeast which still had an undiminished power of fermentation, while the others found no glycogen deposition during the induction period in fresh yeast. No support for Willstätter's hypothesis was obtained, and it must be admitted that the mere fact that glycogen deposition may occur can hardly be advanced as proof that glucose can only be fermented by way of glycogen.

The glycogen content of the bones of rats increases after birth; after eleven to thirteen days there occurs a rapid decline, concomitant with progressive calcification (54). Glycogen appears in developing salmon eggs about midway between the time of fertilization and the time of hatching; it appears in the muscles sooner than in the liver (73). The total carbohydrate (and glycogen) content of developing honey bees increases between the third and sixth days of larval development and decreases subsequently, so that the amounts found at the end of pupal development are very low (106). A decrease in glycogen takes place in sea urchin eggs immediately after fertilization; no lactate is formed and there is no change in phosphate distribution (115).

HORMONES

The mechanisms through which hormones act are not yet understood; a more or less intact cell structure seems to be essential for their action; no clear-cut effect of a hormone on a cell-free enzyme system has as yet been reported. In the opinion of the reviewers, hormones do not participate, like certain vitamins, in the actual enzymatic processes; they are not integral parts of enzyme systems, but are able to affect the rate and direction of enzymatic reactions in the cell.

Epinephrine.—Epinephrine when acting in the intact organism, or on isolated frog muscle, increases the rate of glycogen breakdown, but it has no effect on the cell-free phosphorylase system (28) or on a liver brei (89).

The breakdown of liver glycogen and subsequent rise in blood sugar brought about by epinephrine is not accompanied by the increase in sugar oxidation which accompanies an equal rise in blood sugar brought about by glucose ingestion. Conn *et al.* (24) measured respiratory metabolism for periods of four hours, using a respiration chamber. They found in men that when epinephrine raises the blood-

sugar level to 200 mg. per cent, less glucose is being oxidized than at the fasting blood-sugar level of 80 mg. per cent. When the blood sugar is raised to 350 mg. per cent by epinephrine plus glucose ingestion, no more sugar is oxidized than at a blood-sugar level of 170 mg. per cent after ingestion of glucose alone. At a given blood sugar level they found twice the amount of glucose oxidized after glucose ingestion as after epinephrine injection. Dill *et al.* (38) found that epinephrine injections in normal fasting men either did not change or slightly increased the proportion of carbohydrate oxidized during a period of elevated blood sugar and lactate levels. In men who were exercising Asmussen *et al.* (3) noted some enhancement of glucose oxidation when epinephrine was injected (in one subject the amount of glucose oxidized in three hours rose from 170 to 180, in another from 191 to 225 gm.) ; previously, Courtice *et al.* (1939) had observed no enhanced oxidation of carbohydrate in spite of the long-maintained rise in blood sugar concentration.

Injections of epinephrine into a peripheral vein in dogs produced hyperglycemia, but did not cause an increase in the difference between arterial and venous blood sugar concentration of the leg. The same amount of epinephrine injected into the portal system was followed by hyperglycemia and an arteriovenous blood sugar difference similar to that seen after glucose ingestion. Since most of the epinephrine is destroyed during its passage through the liver, epinephrine action outside this organ is greatly diminished or abolished and the increased blood sugar level, brought about by glycogenolysis in the liver, has its usual effect of increasing the uptake of glucose in the tissues (20).

Griffith *et al.* (61) found that the calorigenic effect of epinephrine parallels the blood sugar rather than the blood lactic acid (61). Larson (88a) measured changes of pH in the blood of cats during constant intravenous injection of epinephrine and found decreases of 0.1 to 0.2 pH units during injection. Serum potassium showed an initial rise and then a decrease; in eviscerated cats an initial rise in serum potassium did not take place. Glycogenolysis in the liver might possibly be responsible for the initial rise in serum potassium, since the reverse process, glycogen storage, is accompanied by a retention of water and potassium ions. The drop in plasma potassium which follows the initial rise is attributed by Larson to a decrease in the pH of the tissues.

Richter (120) found that up to 70 per cent of epinephrine (15 to 20 mg.) after being administered orally to man is excreted as a con-

jugated sulfate. He considers this is a possible physiological mechanism for the inactivation of epinephrine.

Insulin.—Among the well-established effects of insulin are (*a*) increased oxidation of sugar and (*b*) increased deposition of glycogen in muscle. According to the experimental conditions either one or the other of these effects may predominate. It has been shown that both processes can take place in the absence of insulin. Other important effects of insulin are (*c*) inhibition of glycogenolysis in the liver and (*d*) suppression of protein and fat catabolism by an indirect sparing action; this leads to a decrease both of gluconeogenesis from amino acids and of ketone body formation from fatty acids in the liver. Stadie *et al.* (138) found that insulin, in the presence of fructose, fumarate, or lactate, inhibited the formation of ketone bodies in liver slices of diabetic cats.

An effect of insulin on glycogen deposition in isolated mammalian muscle was demonstrated by Gemmill (52). Rat diaphragm was suspended in oxygenated Ringer's solution containing glucose. After three hours the average glycogen content without insulin was 0.44, with insulin 0.69 per cent; without glucose insulin had no effect. It is of interest, in view of the experiments to be discussed next, that Gemmill found insulin to have no effect on the oxygen consumption of the isolated muscle.

Addition of insulin delays the falling off in respiration of minced pigeon breast muscle [Krebs & Eggleston (1938)]. Stadie *et al.* (139) confirmed this effect, but, like Shorr & Barker (1939) found it to be smaller than had originally been noted, and also found it to be independent of the simultaneous addition of citrate. Stare & Baumann (142) observed that the effect of insulin was more pronounced when muscles from pancreatectomized pigeons were used. The relation of this effect to the action of insulin in the intact animal still remains obscure. Insulin stimulates the respiration of baker's yeast also (25).

Soskin & Levine (136) reported that insulin, when injected along with glucose into eviscerated dogs, prevented the disappearance of muscle glycogen. Usually muscle glycogen disappears in such animals when not treated with insulin. In order to maintain the blood sugar at about 200 mg. per cent the insulinized animals required 0.5 gm. of glucose per kilo of body weight per hour, while the noninsulinized animals required only one half as much. It is stated in the conclusion of their paper that the administration of insulin does not increase the

rate of sugar utilization by the extrahepatic tissues. This conclusion was arrived at, because the muscle glycogen which disappeared in the noninsulinized animal was assumed to have been "utilized" (i.e., oxidized) and was therefore added, in a calculation of total utilization, to the amount of glucose injected. It was assumed that in the insulinized animal this oxidation of muscle glycogen is masked by a simultaneous deposition of glycogen. Greeley & Drury (59) conclude, however, that the muscle glycogen which disappears from eviscerated animals seems chiefly to be changed to lactic acid.

Greeley & Drury compared the glucose requirement of rabbits after acute pancreatectomy and evisceration and after pancreatectomy followed a few days later by evisceration. Both types of animals required about 200 mg. of glucose per kilo of body weight per hour for the maintenance of the original blood sugar level, an amount which covers only a sixth to a third of the energy requirements of the animals. These investigators point out that the depancreatized eviscerated animal cannot oxidize glucose as rapidly as the intact animal.

Shorr et al. (129) found that excised cardiac tissue of diabetic dogs regained the capacity to oxidize carbohydrate, when incubated for some time in Ringer's solution containing glucose and phosphate. This interesting phenomenon was attributed to the release of the tissues from inhibitory influences carried over from the intact animal. A similar phenomenon is noted in eviscerated animals when they pass into the second stage; this sets in at variable times after the operation and is characterized by prostration of the animal and a marked increase in the glucose requirement. Greeley & Drury (59) have pointed out that caution is necessary in drawing conclusions as to the glucose utilization rates of eviscerated animals, especially if the animal is already diabetic.

Drury (40) found that when depancreatized rats are kept on a regime of fasting and of feeding a high carbohydrate diet on alternate days, they are unable to maintain their body weight, but when given insulin on the day of feeding, they are able to do so. He argues that an important effect of insulin in the economy of the organism is a storage of sugar as glycogen and as fat. Another possibility is that increased oxidation of sugar in the rats receiving insulin might spare fat and so help maintain weight.

Greeley (58) measured the duration of action of varying doses of insulin (2 to 100 units), injected intravenously into depancreatized dogs, by adjusting the rate of glucose injection in such a manner that

the blood sugar remained constant. This required steadily decreasing rates of glucose injection until a point was reached when without glucose injection the blood sugar began to rise. With this method it was found that the time required for the destruction of insulin in the body was proportional to the logarithm of the dose injected.

Goadby & Richardson (55) measured the disappearance of insulin (40 units) from the blood of rabbits by withdrawing blood at intervals and testing it for its hypoglycemic effect in other rabbits. Exclusion of the liver from the circulation or injection of pituitary extract had no effect on insulin disappearance from the blood, but nephrectomy definitely delayed it. Marble *et al.* (102) and Rushton (123) found that in some instances plasma from diabetics antagonized insulin action. Selye (128) found that while fasting generally increases sensitivity to insulin in the rat, there is a short period after two days of fasting when sensitivity to insulin is very low.

Studies concerned with the determinations of the insulin content of the pancreas have been continued. The insulin content per unit of body weight varies considerably in different species; this value decreases in the following order: mouse, rabbit, chimpanzee, dog, rat, cat, guinea pig (103). In hypophysectomized rats the insulin content of the pancreas was found to be within the normal range (62, 64). A high fat diet lowered the insulin content in the hypophysectomized animals (as it does in controls). One week after return to a balanced diet the insulin content was back to normal. Insulin injections had an even more depressing effect on the insulin content of the pancreas than the fat diet (63). Appel & Hughes (2) made glucose tolerance tests in schizophrenic patients who, for two months, received large doses of insulin daily. During and for three to five months after cessation of the insulin treatment, these patients showed a markedly decreased ability to utilize glucose.

Anterior pituitary and adrenal cortex.—Anterior pituitary extracts exert an effect on carbohydrate metabolism: (*a*) through the adrenals by controlling the production of steroids which act synergistically with certain anterior pituitary factors; (*b*) through the pancreas by affecting chiefly the production of insulin in the islet tissue; and (*c*) through a direct effect which is demonstrable in depancreatized and adrenalectomized animals.

The anterior lobe of the pituitary elaborates a number of specific proteins, the separation of which has presented great difficulties. Young (158) has made fractionations of alkaline extracts of the an-

terior lobe with ammonium sulfate at 0°. The globulin and pseudo-globulin fractions contained the diabetogenic principle. The diabetogenic material still contained the growth principle and also had some thyrotropic, gonadotropic, and anti-insulin activity. It was, however, possible to prepare extracts which had the three latter activities but were not diabetogenic. Dogs injected with diabetogenic material and made temporarily diabetic had normal liver glycogen, high liver fat, and low muscle glycogen.

Greaves *et al.* (57) have described a method for the preparation and assay of the anterior pituitary factor which lowers the respiratory quotient in the glucose-fed rat. Alkaline extraction of the pituitary, followed by repeated salting out with ammonium sulfate between 0.2 and 0.45 saturation, dialysis, and isoelectric precipitation, yielded a protein fraction which was active in a dose of 0.2 mg. per rat, and occasionally preparations were obtained which were active in a dose of 0.08 mg. of protein. Relatively free of thyrotropic, adrenotropic, gonadotropic, lactogenic, and melanophore effects, this material increased growth and was markedly ketogenic. Russell (124) found that saline extracts of the anterior pituitary influenced the disposition of ingested glucose in rats in the following manner: oxidation of glucose was depressed to less than a third of the value found in uninjected controls; deposition of glycogen in the liver was not significantly changed; and deposition of glycogen in the muscles was increased. An extract containing mainly the adrenotropic factor produced similar results. In adrenalectomized rats, adrenal cortical extract depressed glucose oxidation, and increased liver glycogen deposition, but had little effect on muscle glycogen. These results suggested that a synergistic and, in part, a complementary action exists between the cortical hormone and anterior pituitary extracts. Jensen & Grattan (81) have shown that adrenotropic fractions of the anterior lobe have an anti-insulin action and increase the liver glycogen, while thyrotropic, lactogenic, and gonadotropic fractions do not have these effects.

Injection of a saline extract of the anterior lobe of the pituitary led to an increase in the glycogen content in the bodies of fasted mice (110). Saline extracts depressed the blood-sugar level and the nitrogen excretion of fasted normal or adrenalectomized rats, and produced ketosis, while alkaline extracts had no effect on nitrogen metabolism, but also produced ketosis (69).

Marks & Young (104) found that dogs made permanently diabetic by injections of anterior lobe extracts ("Young dogs") differed

from depancreatized dogs by their ability to survive for long periods without insulin therapy. On a protein diet they exhibited hyperglycemia and glycosuria with a D/N ratio of 3.0, and ketonuria; on a diet high in carbohydrates they excreted 85 per cent of the sugar available in the food. A beef suet diet was well tolerated, glycosuria decreased and the glucose tolerance rose. These observations do not favor the hypothesis that fat is converted to sugar in the diabetic organism. Some of Young's older results have been confirmed (95) and although ketosis was as marked in the "Young dog" as in the depancreatized dog, nitrogen excretion was lower. One injection of an anterior lobe extract had no demonstrable effect in normal dogs; however, it markedly aggravated the diabetic condition in depancreatized dogs, necessitating an increase in insulin dosage for a month or more (49).

It is of significance that the different cortical steroids are not of equal potency in their effect on carbohydrate metabolism. According to most investigators desoxycorticosterone is mainly involved in the regulation of electrolyte balance and has little if any direct effect on carbohydrate metabolism. Long *et al.* (93) reported that injection of corticosterone increased the glycosuria of partially depancreatized rats, while the injection of desoxycorticosterone had only a feeble effect. Similar results have been obtained by Ingle (78).

As pointed out by Long *et al.* (93) one of the basic actions of cortical steroids seems to be to increase protein catabolism; this results in increased gluconeogenesis in the liver. It was shown by Grattan & Jensen (56) that corticosterone, 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone in doses of 0.5 mg., protected mice against insulin convulsions and caused an increase in liver glycogen, while desoxycorticosterone, in doses of 2 mg., exhibited no such action. Hartman *et al.* (71) also reported that the protective action of cortical extract is associated with an increase in liver glycogen. In adrenalectomized dogs desoxycorticosterone failed to protect against insulin convulsions, while cortical extract and 17-hydroxycorticosterone were effective (90). In patients with Addison's disease desoxycorticosterone had little if any effect on carbohydrate metabolism, while corticosterone raised the blood sugar level and decreased the R.Q. during fasting and after glucose feeding (148). It is claimed that addition of cortical extract to liver slices *in vitro* inhibits glycogenolysis (127). Those cortical steroids which have the greatest effect on carbohydrate metabolism also appear to have the greatest effect on

muscular efficiency (79). The diminished ability of the muscles of adrenalectomized rats to maintain tension is not due to the poor nutritional state of the animals (154).

A study of the effect of anterior pituitary extract on the insulin content of the pancreas and the morphology of the islet tissue has given interesting results. Only 2.5 units of insulin were found in the pancreas of a "Young dog" as against 76 units in normal controls (105). The simultaneous administration of protamin-Zn-insulin protected the islet tissue against insulin depletion and degenerative changes brought about by injection of anterior pituitary extract (19). Morphological and functional recovery of islet tissue in partially depancreatized cats, made permanently diabetic by injection of anterior pituitary extract, was brought about by administration of insulin (96). In the permanently diabetic dog no such recovery occurred following treatment with insulin.

In addition to the pituitary factor which decreases the insulin content of the dog pancreas, there is also present in crude extracts of the anterior lobe a factor which increases pancreatic insulin. This is best seen in rats, which cannot be made diabetic by injection of pituitary extracts. By suitable separation procedures, this insulin-increasing substance was shown not to be identical with the diabetogenic and growth factors (105).

The carbohydrate metabolism of hypophysectomized rabbits was studied by Greeley (60). He found that it was necessary to supply glucose at rates of 300 to 700 mg. per kg. body weight per hour in order to prevent the blood sugar from dropping to a subnormal level. After glucose had been given for five to six hours, liver and muscle glycogen were found to be low (around 1 and 0.2 per cent respectively). Hepatectomy or evisceration did not materially depress the high glucose requirement of hypophysectomized rabbits. (The average glucose requirement of eviscerated normal rabbits is 200 mg. per kg. per hour.) The author states that the only explanation possible is that the oxidation of glucose in hypophysectomized rabbits is increased. These findings and conclusions are in agreement with older work on hypophysectomized rats.

Several reviews on the relation of the adrenal cortex and the anterior pituitary to carbohydrate metabolism have been published [Long *et al.* (93), Young (157), Rynearson *et al.* (125), Swann (147)]. Shorr (130) has reviewed the relation of hormones to carbohydrate metabolism *in vitro.*

Relation of Fat to Carbohydrate Metabolism

It is assumed at the present time that the chief site of production of blood ketone bodies is the liver, that the long-chain fatty acids yield, by multiple alternate oxidation, more than one equivalent of ketone bodies per molecule, and that the ketone bodies are burned in the extrahepatic tissues. Stadie *et al.* (140) measured oxygen consumption and ketone body production of liver slices from diabetic cats. The molecular ratio of oxygen consumed to ketone bodies formed was close to 1.25, which indicates the formation of four molecules of ketones per molecule of fatty acid oxidized. The nonketone respiration was 22 per cent of the total respiration; allowing for oxidative deamination of amino acids, there was no oxygen available for a conversion of fatty acids to glucose. From these facts Stadie *et al.* (140) concluded that the hypothesis of an overproduction of carbohydrate from fat in diabetes is untenable. Arguments in favor of a conversion of fatty acids to glucose have been advanced by Soskin (137).

A number of authors (9, 32, 141) have estimated that when fat is the main substrate oxidized by the organism, the liver can supply about one half of the basal energy requirements of the tissues in the form of ketone bodies; during exercise this fraction becomes smaller. It is therefore assumed that muscle and other tissues also oxidize fat directly and the low respiratory quotients previously observed for isolated muscle and for hepatectomized animals support this assumption. The diabetic dog can oxidize large amounts of fat completely; this is shown by the observation of Barker (8) that ketosis in depancreatized dogs is not significantly increased when they are made to exercise or when they are injected with dinitrophenol.

The antiketogenic action of glucose and other substances is now usually attributed to a suppression of fat catabolism in the liver rather than to a ketolytic action. Deuel *et al.* (37) have obtained results which, in their interpretation, support a possible ketolytic effect of glucose. They injected *l*-β-hydroxybutyric acid into fed and fasted nephrectomized rats and determined disappearance of the acid by analyzing the entire carcass. They found a more rapid disappearance in fed animals. Mirsky, Nelson & Grayman (1939) who had used the same procedure previously, had found no difference. Deuel states that Mirsky may have carried out the experiments too early after nephrectomy, when carbohydrate oxidation was depressed by the preceding ether anesthesia. Wick *et al.* (151) have claimed for glycine and alanine the same antiketogenic powers as for glucose and even

greater powers for citric acid (99). Somogyi (133) found an increase in blood ketones (up to 3 mg. per cent) during postabsorptive and hyperinsulin hypoglycemia. Longenecker *et al.* (94) showed that rats on a carbohydrate, fat-free diet which contained no thiamin, lost fat from their stores. When thiamin was added to the diet, fat was deposited, indicating that thiamin is involved in the formation of fat from carbohydrate. The fat laid down on the carbohydrate diet contained a large proportion of C_{16} acids.

MISCELLANEOUS

Corkill & Nelson (30) determined the blood sugar in spinal eviscerated cats which had been given insulin and a continuous infusion of glucose at the hourly rate of 120 mg. per kg. of body weight. The average blood sugar found after two hours was 153 mg. per cent. When fructose was added to the glucose so that the total amount of sugar injected was approximately doubled, the blood glucose averaged only 11 mg. per cent. Blatherwick *et al.* (14) found a higher lactic acid content in the livers and muscles of rats after feeding fructose than after feeding glucose.

Darby & Day (34) observed that a diet which contained 30 per cent xylose produced cataract in rats after three weeks; the average blood sugar of these rats was 178 mg. per cent, while on a diet containing the cataractogenic sugar galactose, the average blood sugar was 262 mg. per cent. Arabinose and mannose were not cataractogenic and did not elevate the blood-sugar level. Blatherwick *et al.* (15) found that *d*-mannoheptulose was excreted in the urine after eating avocados. In rats *d*-sorbitol was absorbed at a slow rate and formed no liver glycogen (16).

Cornbleet (31) found that the glucose content of the skin parallels that of the blood, with a lag in its rise and fall. The glucose content of the blood was always above that of the skin; the skin is therefore not a storage organ for sugar.

Kauer & Glenn (82) noted no significant diminution of the blood disastase content in dogs after removal of 84 per cent of the pancreas. Somogyi (134) found that the blood diastase content is low in diabetes; values below 80 units were found in 74 per cent of the diabetics and only in 17 per cent of the normal controls. In a study of the kinetics of the activity of diastase obtained from human urine Somogyi (135) showed that the amount of maltose and glucose formed increased with the enzyme concentration. The products of

breakdown compete with starch for the enzyme; the affinity of the latter is greater for starch and the high-molecular intermediates.

Hart & Lisa (70) determined the fasting blood sugar in 21,000 individuals using the Folin-Wu method and found in 10.5 per cent of the cases a blood-sugar level below 80 mg. per cent; in 7 per cent, the blood sugar concentration was between 79 and 70; in 2.3 per cent, between 69 and 60; in 0.8 per cent, between 59 and 50; in 0.35 per cent, between 49 and 40; and in 0.05 per cent, between 39 and 30 mg. per cent. Wilder & Browne (152) have reviewed the literature pertaining to diabetes mellitus.

LITERATURE CITED

1. ANDERSON, E., AND HERRING, V. V., *Proc. Soc. Exptl. Biol. Med.*, **43**, 363 (1940)
2. APPEL, J. W., AND HUGHES, J., *Am. J. Med. Sci.*, **199**, 829 (1940)
3. ASMUSSEN, E., WILSON, J. W., AND DILL, D. B., *Am. J. Physiol.*, **130**, 600 (1940)
4. ASTBURY, W. T. BELL, F. O., AND HANES, C. S., *Nature*, **146**, 558 (1940)
5. BAER, R. S., AND CORI, C. F. (Unpublished results)
6. BALL, E., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 100 (1939)
7. BANGA, I., OCHOA, S., AND PETERS, R. A., *Biochem. J.*, **33**, 1980 (1939)
8. BARKER, S. B., *J. Physiol.*, **97**, 394 (1940)
9. BARNES, R. H., DRURY, D. R., GREELEY, P. O., AND WICK, A. N., *Am. J. Physiol.*, **130**, 144 (1940)
10. BARRON, E. S. G., AND LYMAN, C. M., *Science*, **92**, 337 (1940)
11. BAUMANN, C. A., AND STARE, F. J., *J. Biol. Chem.*, **133**, 183 (1940)
12. BELITZER, V. A., AND GOLOVSKAYA, K. S., *Science*, **92**, 537 (1940)
13. BELITZER, V. A., AND TSIBAKOVA, E. F., *Biokhimiya*, **4**, 518 (1939)
14. BLATHERWICK, N. R., BRADSHAW, P. J., EWING, M. E., AND SAWYER, S. D., *J. Biol. Chem.*, **136**, 615 (1940)
15. BLATHERWICK, N. R., LARSON, H. W., AND SAWYER, S. D., *J. Biol. Chem.*, **133**, 643 (1940)
16. BLATHERWICK, N. R., BRADSHAW, P. J., EWING, M. E., LARSON, H. W., AND SAWYER, S. D., *J. Biol. Chem.*, **134**, 549 (1940)
17. BOBBIT, B. G., AND DEUEL, JR., H. J., *Am. J. Physiol.*, **131**, 521 (1940)
18. BRUCE, H. M., AND WIEN, R., *J. Physiol.*, **98**, 375 (1940)
19. CAMPBELL, J., HAIST, R. E., HAM, A. N., AND BEST, C. H., *Am. J. Physiol.*, **129**, P328 (1940)
20. CARRASCO-FORMIGUERA, R., *Am. J. Physiol.*, **129**, P330 (1940)
21. CARSON, S. F., AND RUBEN, S., *Proc. Natl. Acad. Sci. U.S.*, **26**, 422 (1940)
22. COLOWICK, S. P., WELCH, M. S., AND CORI, C. F., *J. Biol. Chem.*, **133**, 359 (1940)
23. COLOWICK, S. P., WELCH, M. S., AND CORI, C. F., *J. Biol. Chem.*, **133**, 641 (1940)

24. CONN, J. W., CONN, E. S., AND JOHNSTON, M. W., *J. Nutrition,* Suppl., **19**, 16 (1940)

25. COOK, E. S., WALTER, E. M., AND EILERT, M. R., *Proc. Soc. Exptl. Biol. Med.,* **44**, 547 (1940)

26. CORI, G. T., AND CORI, C. F., *J. Biol. Chem.,* **131**, 397 (1939)

27. CORI, G. T., AND CORI, C. F., *J. Biol. Chem.,* **135**, 733 (1940)

28. CORI, C. F., *Endocrinology,* **26**, 285 (1940)

29. CORI, C. F., *Cold Spring Harbor Symposia Quant. Biol.,* **7**, 260 (1939)

30. CORKILL, A. B., AND NELSON, J. F., *Australian J. Exptl. Biol. Med. Sci.,* **18**, 171 (1940)

31. CORNBLEET, T., *Arch. Dermatol. Syphilol.,* **41**, 193 (1940)

32. CRANDALL, JR., L. H., IVY, H. B., AND ELMI, G. J., *Am. J. Physiol.,* **131**, 10 (1940)

33. DAHL, O., *Z. physiol. Chem.,* **263**, 81 (1940)

34. DARBY, W. J., AND DAY, P. L., *J. Biol. Chem.,* **133**, 503 (1940)

35. DAVIDSON, J. N., AND GARRY, R. C., *J. Physiol.,* **97**, 509 (1940)

36. DELRUE, G., AND DEKEYSER, J., *Compt. rend. soc. biol.,* **133**, 709 (1940)

37. DEUEL, JR., H. J., HALLMAN, L. F., GREELEY, P. O., BUTTS, J. S., AND HALLIDAY, N., *J. Biol. Chem.,* **133**, 173 (1940)

38. DILL, D. B., JOHNSON, R. E., AND DALY, C., *Am. J. Med. Sci.,* **198**, 702 (1939)

39. DISCHE, Z., *Compt. rend. soc. biol.,* **133**, 380 (1940)

40. DRURY, D. R., *Am. J. Physiol.,* **131**, 536 (1940)

41. EILER, J. J., STOCKHOLM, M., AND ALTHAUSEN, T. L., *J. Biol. Chem.,* **134**, 283 (1940)

42. EULER, H. V., AND HÖGBERG, B., *Naturwissenschaften,* **28**, 79 (1940); *Z. physiol. Chem.,* **263**, 49 (1940)

43. EVANS, JR., E. A., *Biochem. J.,* **34**, 829 (1940)

44. EVANS, JR., E. A., AND SLOTIN, L., *J. Biol. Chem.,* **136**, 301 (1940)

45. FENN, W. O., AND HAEGE, L. F., *J. Biol. Chem.,* **136**, 87 (1940)

46. FERDMAN, D. L., AND EPSTEIN, S. F., *Science,* **91**, 365 (1940)

47. FERREBEE, J. W., *J. Biol. Chem.,* **136**, 719 (1940)

48. FLOCK, E. V., AND BOLLMAN, J. L., *J. Biol. Chem.,* **136**, 469 (1940)

49. GAEBLER, D. H., AND GALBRAITH, H. W., *J. Biol. Chem.,* **133**, xxxv (1940)

50. GEIGER, A., *Biochem. J.,* **34**, 465 (1940)

51. GEMMILL, C. L., *Cold Spring Harbor Symposia Quant. Biol.,* **7**, 216 (1939)

52. GEMMILL, C. L., *Bull. Johns Hopkins Hosp.,* **66**, 232 (1940)

53. GEMMILL, C. L., *Bull. Johns Hopkins Hosp.,* **66**, 71 (1940)

54. GLOCK, G. E., *J. Physiol.,* **98**, 1 (1940)

55. GOADBY, H. K., AND RICHARDSON, J. S., *J. Physiol.,* **97**, 417 (1940)

55a. GODA, T., *Biochem. Z.,* **298**, 431 (1938)

56. GRATTAN, J. F., AND JENSEN, H., *J. Biol. Chem.,* **135**, 511 (1940)

57. GREAVES, J. D., FREIBERG, I. K., AND JOHNS, H. E., *J. Biol. Chem.,* **133**, 243 (1940)

58. GREELEY, P. O., *Am. J. Physiol.,* **129**, 17 (1940)

59. GREELEY, P. O., AND DRURY, D. R., *Am. J. Physiol.,* **130**, 249 (1940)

60. GREELEY, P. O., *Endocrinology,* **27**, 317 (1940)

61. GRIFFITH, JR., F. R., EMERY, F. E., AND LOCKWOOD, J. E., *Am. J. Physiol.,* **128**, 281; **130**, 197 (1940)

62. GRIFFITHS, M., AND YOUNG, F. G., *Nature,* **146,** 266 (1940)
63. HAIST, R. E., AND BEST, C. H., *Science,* **91,** 410 (1940)
64. HAIST, R. E., *J. Physiol.,* **98,** 419 (1940)
65. HALL, W. K., DOTY, J. R., AND EATON, A. G., *Am. J. Physiol.,* **131,** 252 (1940)
66. HANES, C. S., *Nature,* **145,** 348 (1940)
67. HANES, C. S., *Proc. Roy. Soc. (London),* B, **128,** 421 (1940)
68. HANES, C. S., *Proc. Roy. Soc. (London),* B, **129,** 174 (1940)
69. HARRISON, H. C., AND LONG, C. N. H., *Endocrinology,* **26,** 971 (1940)
70. HART, J. F., AND LISA, J. R., *Endocrinology,* **27,** 19 (1940)
71. HARTMAN, F. A., BROWNELL, K. A., WALTHER, R., AND EDELMANN, A., *Endocrinology,* **27,** 642 (1940)
72. HASTINGS, A. B., KISTIAKOWSKY, G. B., CRAMER, R. D., KLEMPERER, F. W., SOLOMON, A. K., AND VENNESLAND, B., *Science,* **91,** 421 (1940)
73. HAYES, F. R., AND HOLLETT, A., *Can. J. Research, D,* **18,** 53 (1940)
74. HERBERT, D., GORDON, H., SUBRAHMANYAN, V., AND GREEN, D. E., *Biochem. J.,* **34,** 1108 (1940)
75. HILLS, G. M., *Biochem. J.,* **34,** 1070 (1940)
76. HOLMES, B. E., *Biochem. J.,* **34,** 926 (1940)
77. HUSZÁK, I., *Biochem. Z.,* **303,** 349 (1940)
78. INGLE, D. J., *Proc. Soc. Exptl. Biol. Med.,* **44,** 176 (1940)
79. INGLE, D. J., AND KENDALL, E. C., *Proc. Soc. Exptl. Biol. Med.,* **45,** 602 (1940)
80. IRI, S., *J. Biochem.,* **30,** 217 (1939)
81. JENSEN, H., AND GRATTAN, J. F., *Am. J. Physiol.,* **128,** 270 (1939–40)
82. KAUER, J. T., AND GLENN, F., *Am. J. Physiol.,* **131,** 437 (1940)
83. KERLY, M., AND BOURNE, M. C., *Biochem. J.,* **34,** 563 (1940)
84. KJERULF-JENSEN, K., AND LUNDSGAARD, E., *Z. physiol. Chem.,* **266,** 216 (1940)
85. KOBAYASI, T., *J. Biochem.,* **32,** 289 (1940)
86. KREBS, H. A., AND EGGLESTON, L. V., *Biochem. J.,* **34,** 442 (1940)
87. KREBS, H. A., *Biochem. J.,* **34,** 775 (1940)
88. LARSON, H. W., BLATHERWICK, N. R., BRADSHORE, P. J., EWING, M. E., AND SAWYER, S. D., *J. Biol. Chem.,* **136,** 1 (1940)
88a. LARSON, P. S., *Am. J. Physiol.,* **129,** P401; **130,** 562 (1940)
89. LEE, M., AND RICHTER, D., *Biochem. J.,* **34,** 551 (1940)
90. LEWIS, R. A., KULMAN, D., DELBUE, C., KOEPF, G. F., AND THORN, G. W., *Endocrinology,* **27,** 971 (1940)
91. LIPMANN, F., *J. Biol. Chem.,* **134,** 463 (1940)
91a. LIPMANN, F., *Cold Spring Harbor Symposia Quant. Biol.,* **7,** 248 (1939)
92. LIPTON, M. A., AND ELVEHJEM, C. A., *Cold Spring Harbor Symposia Quant. Biol.,* **7,** 184 (1939)
93. LONG, C. N. H., KATZIN, B., AND FRY, E. G., *Endocrinology,* **26,** 309 (1940)
94. LONGENECKER, H. E., GEVIN, G., AND McHENRY, E. W., *J. Biol. Chem.,* **134,** 693 (1940)
95. LOUBATIÈRES, A., *Compt. rend. soc. biol.,* **133,** 26 (1940)
96. LUKENS, F. D. W., AND DOHAN, F. C., *Science,* **92,** 222 (1940)
97. LUTWAK-MANN, C., *Biochem. J.,* **34,** 517 (1940)

98. MacKay, E. M., Wick, A. N., and Carne, H. O., *J. Biol. Chem.,* 132, 613 (1940)

99. MacKay, E. M., Carne, H. O., and Wick, A. N., *J. Biol. Chem.,* 133, 59 (1940)

100. Macleod, J., *Am. J. Physiol.,* 129, P413 (1940)

101. Marazzi, R., *Am. J. Physiol.,* 131, 36 (1940)

102. Marble, A., Fernald, A. T., and Smith, R. M., *Endocrinology,* 26, 735 (1940)

103. Marks, H. P., and Young, F. G., *Nature,* 146, 31 (1940)

104. Marks, H. P., and Young, F. G., *J. Endocrinol.,* 1, 470 (1939–40)

105. Marks, H. P., and Young, F. G., *Lancet,* I, 493 (1940)

106. Melampy, R. M., and Olsan, R. D., *Proc. Soc. Exptl. Biol. Med.,* 45, 754 (1940)

107. Meyerhof, O., and Perdigon, E., *Enzymologia,* 8, 353 (1940)

108. Needham, J., Lehmann, H., and Nowinski, W. W., *Compt. rend. soc. biol.,* 133, 6 (1940)

109. Nelson, D., *Am. J. Physiol.,* 129, P429 (1940)

110. Neufeld, A. H., Scoggam, S. M., and Stewart, G. S., *Endocrinology,* 27, 132 (1940)

111. Newman, H. W., Van Winkle, Jr., W., Kennedy, N. K., and Morton, M. C., *J. Pharmacol.,* 68, 194 (1940)

112. Ochoa, S., *Nature,* 145, 747; 146, 267 (1940)

113. Ochoa, S. (Personal communication)

114. Ochoa, S., and Rossiter, R. J., *J. Physiol.,* 97, 1P (1940)

115. Örström, Å., and Lindberg, O., *Enzymologia,* 8, 367 (1940)

115a. Peters, R. A., *Nature,* 146, 387 (1940)

116. Phillips, R. A., and Gilder, H., *Endocrinology,* 27, 601 (1940)

117. Potter, V. R., *J. Biol. Chem.,* 134, 417 (1940)

118. Potter, V. R., *Medicine,* 19, 441 (1940)

119. Reiser, R., *J. Biol. Chem.,* 135, 303 (1940)

120. Richter, D., *J. Physiol.,* 98, 361 (1940)

121. Rudney, H., *Science,* 92, 112 (1940)

122. Runnström, J., Sperber, E., and Bárány, E., *Nature,* 145, 106 (1940)

123. Rushton, J. G., *Proc. Staff Meetings Mayo Clinic,* 15, 417 (1940)

124. Russell, J. A., *Am. J. Physiol.,* 128, 552 (1939–40)

125. Rynearson, E. H., and Schweizer, L. R., *Arch Internal Med.,* 66, 226 (1940)

126. Sacks, J., *Am. J. Physiol.,* 129, 227 (1940)

127. Seckel, H. P. G., *Endocrinology,* 26, 97 (1940)

128. Selye, H., *Endocrinology,* 27, 434 (1940)

129. Shorr, E., Barker, S. B., Cohen, E., and Malam, M., *Am. J. Physiol.,* 129, P463 (1940)

130. Shorr, E., *Cold Spring Harbor Symposia Quant. Biol.,* 7, 323 (1939)

131. Smyth, D. H., *Biochem. J.,* 34, 1046 (1940)

132. Sober, H. A., Lipton, M. A., and Elvehjem, C. A., *J. Biol. Chem.,* 134, 605 (1940)

133. Somogyi, M., *Proc. Soc. Exptl. Biol. Med.,* 45, 644 (1940)

134. Somogyi, M., *J. Biol. Chem.,* 134, 315 (1940)

135. SOMOGYI, M., *J. Biol. Chem.*, **134**, 301 (1940)
136. SOSKIN, S., AND LEVINE, R., *Am. J. Physiol.*, **129**, 782 (1940)
137. SOSKIN, S., *Endocrinology*, **26**, 297 (1940)
138. STADIE, W. C., LUKENS, F. D. W., AND ZAPP, JR., J. A., *J. Biol. Chem.*, **132**, 393 (1940)
139. STADIE, W. C., ZAPP, JR., J. A., AND LUKENS, F. D. W., *J. Biol. Chem.*, **132**, 411 (1940)
140. STADIE, W. C., ZAPP, JR., J. A., AND LUKENS, F. D. W., *J. Biol. Chem.*, **132**, 423 (1940)
141. STADIE, W. C., *J. Clin. Investigation,* **19**, 843 (1940)
142. STARE, F. J., AND BAUMANN, C. A., *Cold Spring Harbor Symposia Quant. Biol.,* **7**, 227 (1939) ; *J. Biol. Chem.,* **133**, 453 (1940)
143. STERN, K., AND MELNICK, J. L., *J. Biol. Chem.*, **135**, 365 (1940)
144. STRAUB, F. B., *Biochem. J.*, **34**, 483 (1940)
145. SÜLLMANN, H., *Helv. Chim. Acta*, **23**, 605 (1940)
146. SÜLLMANN, H., AND BRÜCKNER, R., *Enzymologia,* **8**, 167 (1940)
147. SWANN, H. G., *Physiol. Rev.*, **20**, 493 (1940)
148. THORN, G. W., KOEPF, Z. F., LEWIS, R. A., AND OLSEN, E. F., *J. Clin. Investigation,* **19**, 813 (1940)
149. VERZÁR, F., *Die Funktion der Nebennierenrinde* (Basel, 1939)
150. WARREN, C. O., *Am. J. Physiol.*, **128**, 455 ; **131**, 176 (1940)
151. WICK, A. N., MACKAY, E. M., CARNE, H. O., AND MAYFIELD, H. M., *J. Biol. Chem.,* **136**, 237 (1940)
152. WILDER, R. M., AND BROWNE, H. C., *Arch. Internal Med.*, **65**, 390 (1940)
153. WILLSTÄTTER, R., AND RHODEWALD, M., *Enzymologia,* **8**, 1 (1940)
154. WINTER, C. A., AND KNOWLTON, G. C., *Am. J. Physiol.*, **131**, 465 (1940)
155. WOOD, H. G., AND WERKMAN, C. H., *Biochem. J.*, **34**, 129 (1940)
156. WOOD, H. G., WERKMAN, C. H., HEMINGWAY, A., AND NIER, A. O., *J. Biol. Chem.,* **135**, 789 (1940)
157. YOUNG, F. G., *Endocrinology,* **26**, 345 (1940)
158. YOUNG, F. G., *J. Endocrinol.*, **1**, 339 (1939–40)

ADDENDUM

BANGA, I., OCHOA, S., AND PETERS, R. A., *Biochem. J.*, **33**, 1109 (1939) ; *Ann. Rev. Biochem.,* **9**, 270 (1940)
COURTICE, F. C., DOUGLAS, C. G., AND PRIESTLEY, J. G., *Proc. Roy. Soc. (London), B,* **127**, 288 (1939) ; *Ann. Rev. Biochem.,* **9**, 260 (1940)
KALCKAR, H., *Enzymologia,* **2**, 47 (1937) ; *Ann. Rev. Biochem.,* **8**, 67 (1939)
KALCKAR, H., *Biochem. J.*, **33**, 631 (1939) ; *Ann. Rev. Biochem.,* **9**, 268 (1940)
KIESSLING, W., *Biochem. Z.*, **302**, 50 (1939) ; *Ann. Rev. Biochem.,* **9**, 679 (1940)
KREBS, H. A., AND EGGLESTON, L. V., *Biochem. J.*, **32**, 913 (1938) ; *Ann. Rev. Biochem.,* **8**, 220 (1939)
KRUYK, K., AND KLINGMÜLLER, V., *Biochem. Z.*, **300**, 343 (1939) ; *Ann. Rev. Biochem.,* **9**, 267 (1940)
LASZT, L., AND SÜLLMANN, H., *Biochem. Z.*, **278**, 401 (1935) ; *Ann. Rev. Biochem.,* **5**, 206 (1936)

LUNDSGAARD, E., *Z. physiol. Chem.,* **261,** 193 (1939) ; *Ann. Rev. Biochem.,* **9,**
    254 (1940)
MACFARLANE, M. G., *Biochem. J.,* **33,** 565 (1939) ; *Ann. Rev. Biochem.,* **9,** 267
    (1940)
MIRSKI, A., AND WERTHEIMER, E., *Enzymologia,* **7,** 58 (1939) ; *Ann. Rev.*
    Biochem., **9,** 267 (1940)
MIRSKY, I. A., NELSON, N., AND GRAYMAN, I., *J. Biol. Chem.,* **130,** 179 (1939) ;
    Ann. Rev. Biochem., **9,** 242 (1940)
OCHOA, S., PETERS, R. A., AND STOCKTON, L. A., *Nature,* **144,** 750 (1939) ;
    Ann. Rev. Biochem., **9,** 268 (1940)
SHORR, E., AND BARKER, S. B., *Biochem., J.,* **33,** 1798 (1939) ; *Ann. Rev. Bio-*
    chem., **9,** 270 (1940)

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FAT METABOLISM

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In contrast to previous reviews on this subject the present one has of necessity been limited in its scope because of the unavailability of a considerable number of foreign journals. In some instances the abstracts of these foreign papers have been sufficiently complete to justify the inclusion of the work described, but in other cases the abstracter has either failed to mention the analytical method employed or has neglected to include sufficient data to warrant the incorporation of the summarized investigation.

Absorption.—Frazer (1) has reviewed this important phase of fat metabolism and has given emphasis to his view that digestion is not necessarily a requisite for absorption and that unhydrolyzed fat may enter the body by way of the lymph stream, whereas the hydrolyzed products pass immediately into the portal system. While he has undoubtedly observed a difference between the chylomicron count when neutral fat was fed and that observed when the products of digestion were administered, his conclusions are at such variance with those made from convincing chemical data obtained in the classical experiments in which mono- and diglycerides, as well as other esters, had been administered that for the present at least his theory should not be advanced beyond the hypothetical stage.

Freeman & Johnson (2) have confirmed the existence of a hemolytic agent in thoracic lymph which is present only when fats are being absorbed. After ruling out cholesterol, neutral fat, bile salts, and enzymes, as well as changes in pH and osmotic pressure, the conclusion was made that the hemolysis was caused by the soaps and fatty acids. In order to avoid hemolysis as much as possible the soaps and fatty acids enter by way of the lymph stream and are thus diluted to a large extent before entering the general circulation. According to Frazer's ideas these split products pass directly into the blood stream.

Few data relating to the mode of absorption proposed by Verzár have been recorded this year. The report of Macheboeuf & Perrimond-Trouchet (3), that the soluble compounds formed from the combination of fatty acids and glycocholic acid do not pass through collodion

181

membrances at pH of 6.3, again raises the question of the necessity of bile salts for normal fat absorption. It should, however, be emphasized that a collodion membrane is not a living tissue.

The relationship between phosphate and fat absorption has not been clarified. An increase in phosphatase activity in the small intestine of the white rat (4) and a rise in inorganic and ester phosphate of the intestinal mucosa of swine during fat absorption (5) have been observed.

Weil & Russel (6) have developed a suitable method for determining phosphatase activity in the plasma of rats and report that this activity is markedly reduced by fasting. Neither dietary carbohydrate nor protein increases the fasting activity. On the other hand, feeding of the fraction of the diet which is soluble in alcohol-ether is followed by a marked increase, indicating that the lipids are associated with this rise. Additional studies showed that this change in activity is brought about by certain unsaturated fatty acids such as oleic, linoleic, and erucic. Unsaturated acids containing three, four, five, or eleven carbon atoms and the common saturated ones are without influence, and elaidic acid, the stereoisomer of oleic acid, caused only a slight increase. It is of additional interest to note that whereas cephalin is quite effective, lecithin is devoid of any influence. The suggestion is made that a difference may exist between the metabolism of saturated and unsaturated fatty acids.

Deuel *et al.* (7), employing a new procedure, find the same rates of absorption for partially hydrogenated cottonseed oil, butter fat, or coconut oil when calculated on the basis of body surface area. Triacetin and tributyrin were rapidly absorbed, tricaproin and tricaprylin passed through at a somewhat slower rate, while trilaurin was only slowly removed. Triglycerides containing fatty acids having an odd number of carbon atoms were absorbed more slowly than those containing the naturally occurring acids (8).

Deposition and interconversion of fatty acids.—Progress has again been made by tagging lipids with deuterium. Sperry and co-workers (9), who fed rats linseed oil that had been partially reduced with deuterium, report that whereas only traces of labeled fatty acids could be isolated from the brain, appreciable amounts were found in the liver, intestinal, and depot fats. They thus demonstrated anew the stability of the lipids of the brain. The authors further conclude (10) that the replacement of lipids in the nonsaponifiable fraction of the brain takes place very slowly: in the course of a week only 20 per

cent of the fatty acids in the brain were replaced. During myelination (11) the unsaponifiable lipids and fatty acids are rapidly replaced from the fifteenth to the nineteenth day of extrauterine life. In another communication (12) it was suggested that the alcohol fraction of the nonsaponifiable matter may possibly serve as an intermediate in the synthesis of fatty acids. Such a view is in line with observations made by Schoenheimer and his group (13, 14), who demonstrated that dietary deuteriopalmitic acid is in part deposited as such and partly transformed into cetyl alcohol, stearic acid, palmitoleic acid, and acids of lower molecular weight. Similarly labeled cetyl alcohol was found to be converted in part into palmitic acid and to some extent into stearic. This striking demonstration is indeed far-reaching in its significance. In this connection Lang (15, 16, 17) has shown that liver and muscle contain a fatty acid dehydrogenase the activity of which increases with the length of the carbon chain of the substrate. It has little effect on the unsaturated acids and appears to be specific for the higher fatty acids, forming unsaturated ones therefrom. The codehydrogenase in muscle was shown to be adenylic acid. Inosinic acid and adenosinetriphosphoric acid are equally effective. Bernhard & Schoenheimer (18) fed mice a low-fat diet (bread and heavy water) and again demonstrated a failure of the synthesis of fatty acids less saturated than oleic; they submit further evidence (19) for their belief that the regeneration of fatty acids takes place most rapidly in the liver. According to Hilditch & Pedelty (20), no marked preferential utilization of any particular fatty acid occurs during starvation of the pig, although there seems to be a tendency in this direction in the removal of oleic acid during the later stages. These investigators likewise present further evidence (21) for Hilditch's view that the stearo-glycerides may arise as a result of the saturation of oleo-glycerides. In a study of the soft pork problem (22) the high inverse correlation between the degree of firmness of the fat and the amount of peanut oil or other softening oils in the diet has been reconfirmed. Although the addition of cottonseed meal to such rations resulted in an increase in firmness, the conclusion was made that no particular advantage resulted therefrom.

Essential fatty acids.—The question of the superiority of arachidonic acid over linoleic in restoring the deficiency of the fat-free diets of rats still remains a controversial one. Hume *et al.* (23) believe that although methyl arachidonate may not outrank methyl linolate with respect to its activity in curing the skin lesions, it is nevertheless the

more efficient promoter of growth. Burr and co-workers (24) continue with their belief that arachidonic acid is not superior in any of its effects. Data obtained with radioactive phosphorus administered to rats on fat-free diets (25) indicate that the administration of essential fatty acids does not influence the turnover of phospholipids in the liver or kidney. On the other hand, the turnover in the muscles was approximately one-third larger in animals not receiving essential acids. This observation adds weight to Burr's belief that in this fat-deficiency disease, fat is utilized to a greater extent than in the normal animal.

The cure of the abnormal dermal condition in vitamin-B_6 deficiency by essential fatty acids has again been reported by Steenbock and associates (26). The action has been shown to be independent of vitamin B_6. The acrodynia, as this dermal abnormality is known, can also be cured by a rice-bran concentrate. This latter action has been found to be entirely independent of the fatty acids but is dependent upon B_6 plus a second factor present in rice-bran concentrates. Burr's deficiency disease can likewise be cured by a rice-bran concentrate.

Ketosis.—The relationship between ketosis and carbohydrate metabolism has again been emphasized. The fasting ketosis of male rats, which increases less rapidly with increase of age (27), has been ascribed to the slower disappearance of glycogen from the livers of older rats. β-Hydroxybutyric acid, intraperitoneally administered, disappeared more slowly from the tissues of nephrectomized rats that had been starved than from those of rats which had received glucose (28). Choline (29), which causes an increase in liver glycogen, diminishes the ketonuria produced by the administration of phlorhizin. Endogenous ketosis, produced by fasting, by administration of anterior pituitary extracts, or by phlorhizin, occurs in the liver; the ketone bodies thus produced are utilized by the muscles (30).

Liver.—The role of the liver in lipid metabolism continues to be investigated extensively. Johnston, Irvin & Walton (31) found only small amounts of free choline in hepatic and gall bladder bile of man, hog, and dog, but detected large amounts of combined choline in both fluids. Their clear-cut demonstration of the presence of lecithin in bile is contrary to the report of Jones & Sherberg (32) that lecithin is either entirely absent from dog or hog bile or is present only in traces. Choline (33) prevents the accumulation of fat in the livers of rats poisoned with carbon tetrachloride as well as in dogs in which the

pancreatic duct has been ligated (34). It is believed that the liver damage caused by carbon tetrachloride is due to a decrease in the oxidation of fatty acids and an increased destruction or decreased synthesis of cholesterol. Choline also causes a distinct decrease in the phospholipid content of the livers of dogs in which fatty livers had been produced by the administration of phlorhizin (35). In contrast to mammals, ducks and pigeons fail to develop fatty livers on diets high in fat and free from choline, nor do they deposit an excess of fat in the livers after the administration of anterior pituitary extracts (36).

Dragstedt has summarized his ideas on the current status of lipocaic (37) and re-emphasizes his view that it is a second hormone present in the internal secretion of the pancreas. The criticism that has been made of Dragstedt's work that the mere histological examination of the liver is not a sufficiently accurate method for ascertaining the lipotropic action of lipocaic preparations was answered in his previous study (38), in which it was shown that a very close parallelism exists between lipid content as determined by histological examination and by actual chemical analysis. Dragstedt's observation that the administration of as little as 60 to 100 mg. of a dried pancreatic extract produces a typical effect is submitted as further evidence that lipocaic is not choline or lecithin. He also supports his view that lipocaic is an internal secretion by citing anew his previous observations that dogs showing signs of lipocaic deficiency are not restored to a normal state by the administration of pancreatic juice (38).

On the other hand, Montgomery, Entenman, Gibbs & Chaikoff (39), who, for a period of twenty weeks, fed pancreatic juice obtained from normal dogs to depancreatized dogs receiving insulin and an adequate diet, failed to produce fatty livers. Thus, they do not support Dragstedt in his view that the factor is unrelated to the external secretion of the pancreas. The reviewer, however, must point out that previous to the administration of the pancreatic juice all of Chaikoff's dogs received raw pancreas twice daily. This resulted in a vigorous appetite. According to Dragstedt, a loss in appetite and decreased activity are symptoms which are associated with lipocaic deficiency. It is therefore debatable whether Chaikoff's dogs were properly prepared for the investigation.

Information on the chemical nature of lipocaic remains meager. It is ultrafilterable, is not related to proteins, polypeptides, or lipids, and is not destroyed when heated for a short time with $0.01 N$ sodium carbonate (40).

McHenry & Gavin (41) find that the production of fatty livers observed when an extract of beef liver is fed to rats on vitamin-B-deficient diets can be prevented by the administration of lipocaic but not by choline, thus further differentiating between the two. These investigators propose a method for the assay of lipocaic preparations based on these findings. Neither choline nor lipocaic prevents the accumulation of liver fat which results from the administration of thiamin to vitamin-B_1-deficient chicks or rats (42). Additional evidence is presented in favor of the hypothesis of McHenry that vitamin B_1 is necessary for the synthesis of fat from carbohydrate (43).

Chaikoff and associates have made further use of radioactive phosphorus to study phospholipid metabolism and report (44) that cystine and cysteine, like methionine and choline, stimulate the rate of phospholipid turnover in the livers of rats within six hours after single feedings of the amino acids. It is now generally recognized that whereas methionine and choline exert a lipotropic action on the livers of rats on diets low in protein and high in fat, cystine and cysteine, on the other hand, cause an increase in liver fat. It would seem possible therefore that the effect on phospholipid turnover is not related to lipotropic activity. On the other hand, as the authors themselves point out, the conditions obtaining in these experiments differ from the usual technique for determining lipotropic activity. It is evident that more work is desirable on this question. This same group (45) also reports that glycine, alanine, serine, tyrosine, proline, glutamic acid, and asparagine do not influence the turnover and that taurine, creatine, sarcosine, and di(β-hydroxyethyl) sulfoxide are likewise without effect.

Singal (46) has continued his studies of the influence of cystine and related substances on the lipid content of the livers of mice on diets low in protein and high in fat, and has demonstrated that the betaine of cystine, unlike cystine, exerts a lipotropic action whereas cystine disulfoxide resembles cystine in its behavior. The next higher homologues of cystine, dl-pentocystine and dl-hexocystine, were found to be entirely without influence on liver fat. S-methylcystine and taurine were also found to be ineffective (47), as were the betaines of serine, threonine, and allothreonine (48).

Best & Ridout (49) fed the unnatural form of methionine as well as the racemic mixture and observed that both products were as effective in preventing the accumulation of fat in the livers of rats as was an equivalent amount of l-methionine. These workers likewise

reported that their basal diet, supplemented with as much as 1 per cent of *dl*-methionine, was no more effective than when only 0.125 per cent was added. Unfortunately no information is given on the methionine content of their basal diet and hence it is difficult to evaluate their data so far as methionine requirements are concerned.

Best & Ridout, as well as Channon and his co-workers, are of the opinion that constituents of the protein molecule other than cystine and methionine are concerned with the production of fatty livers of the type referred to. The data presented by the first group are subject to criticism, as was mentioned above, because of their failure to report the composition of the basal diet. Channon *et al.* (47) are now studying the effects of different fractions of protein hydrolyzates and have obtained results which, they claim, justify their contention. No data, however, are included.

Of the other naturally occurring amino acids, tyrosine alone has been found to be effective and its action was reported as being very slight (50). As has already been mentioned, Chaikoff found that this particular amino acid was inert so far as phospholipid turnover is concerned. Here again a lack of correlation between lipotropic activity and phospholipid turnover is evident. The demonstration by Singal & Eckstein (51) that arachin, which contains 5.5 per cent tyrosine but only small amounts of methionine (0.5 per cent), was no more lipotropic at a 20 per cent level than at 5 per cent suggests either that tyrosine when in the protein molecule may be ineffective or that its action may be inhibited by some other constituent. The group at Michigan (51, 52, 53, 54) have demonstrated a lipotropic action whenever the methionine content of the supplemented low protein-high fat diet was equivalent to that of a 20 per cent casein diet regardless of the protein employed (casein, edestin, gliadin, or arachin). On the other hand, inconsistent results were obtained (53) in so far as supplementary cystine failed to increase the fat content of the livers when gliadin was employed as the protein in the basal diet. It was suggested that this failure might be due to a possible inhibitory effect of some other amino acid on the action of cystine. It is evident that more work is needed on these questions.

Entenman, Lorenz & Chaikoff have further investigated the lipid metabolism in the fowl (55). The hatched chick was found to have a lipemia (1000 mg. per cent) and a fatty liver containing very large amounts of cholesterol, mainly in the esterified form. At hatching time the yolk sacs contained large amounts of neutral fat and consider-

able amounts of phospholipids and cholesterol. Absorption from the sac was practically complete after five days, the neutral fat fraction disappearing more rapidly than the others. The lipid content of the blood and liver fell rapidly after the second day, the decline being due primarily to a loss in cholesterol esters and total fatty acids. The phospholipids showed no significant changes. The mechanism whereby the fatty livers and lipemias are produced is discussed and the suggestion is made that the high content of cholesterol in the yolk contributes to both of these conditions.

Blood lipids.—The cholesterol content of the human aorta and of that of cattle increases with age while the mean values for phospholipids remain practically unchanged (56). These findings are in accordance with the older observations that in arteriosclerosis, a disease of old age, the blood cholesterol values are above normal. This has been confirmed (57) and the suggestion has been made that this offers a means of distinguishing between the condition known as arteriosclerosis and thromboangiitis obliterans, since in the latter case the level of the blood lipids is normal. In Addison's disease both the free cholesterol and cholesterol ester content (58) of the serum invariably increase while the total lipids fluctuate above and below normal. The administration of cortical hormone restores the serum lipids to normal. Gildea, Mann & Peters (59) believe that the level of serum cholesterol, phospholipids, and neutral fat is dependent upon the amount of thryoid hormone present in the body at any particular time and they present clinical evidence in support of their view. In myxedema the serum lipid content is high (total cholesterol values ranging from 335 to 603 mg. per cent) and falls after medication with desiccated thyroid. The same investigators (60) have made an extensive study of the serum lipids of forty-three hyperthyroid patients; they arrive at the conclusion that the lipid level before iodine therapy is of little value in predicting the degree of improvement following such therapy. Pre-therapeutic values were below normal; after iodine treatment a rise was usually observed. The cholesterol values likewise rose after thyroidectomy. Fleischmann, Shumacker & Wilkins (61) agree that the thyroid is a regulator of the blood cholesterol level. Their data on thyroidectomized rabbits are quite similar to certain observations made upon hypothyroid children. In both cases the serum cholesterol was definitely above normal. Marked spontaneous fluctuations in the levels for individual rabbits and children were observed which were in marked contrast to the tendency towards a uniformity for normal

rabbits and healthy children. The sharp rise following the operation in the rabbits was likewise similar to the one observed in the disease after withdrawal of thyroid medication. Thyroidectomized rabbits and hypothyroid children both showed marked sensitivity to a single injection of thyroxine, the effect being a drop in the serum cholesterol.

The view that the cephalins in contrast to the lecithins are non-metabolic in nature is becoming more generally accepted. Rubin (62) observed that the level of the corpuscular lipids remains practically unaltered during the lipemia of the diabetic child, and also when the level of the lipids of the whole blood of dogs is increased by a fatty meal. In this connection it will be recalled that the predominating phospholipid in the erythrocytes has been shown to be cephalin. Furthermore, Artom & Freeman (63) reported that an increase in the blood lecithin content of rabbits fed considerable amounts of fat was usually observed whenever a definite lipemia was produced. The cephalin levels on the other hand not only failed to rise but usually dropped. Much of the lipid fraction of blood stroma and erythrocytes which was formerly included in the neutral fat fraction appears to be present principally in the form of cerebrosides (64). In pernicious anemia and in diabetic lipemia this fraction increases in the erythrocytes. Negligible amounts are found in normal plasma. Entenman, Changus, Gibbs & Chaikoff (65) investigated the changes in the blood levels of cholesterol, total fatty acids, and phospholipids during fasting and chronic undernutrition of adult dogs. Generally speaking there were no significant changes in any of the blood lipid fractions in the animals fasted from four to thirty days nor in those in which the chronic undernutrition resulted in a loss of as much as 50 per cent of the body weight during several months. The claim that a lipemia occurs after a prolonged fast in dogs has thus not been confirmed. Voris, Ellis & Maynard (66) have developed a method for determining glycerol in the neutral fat of blood and have applied it in a continuation of their study of the blood precursors of milk fat. The results obtained indicate a removal of neutral fat from the blood by the mammary gland during lactation, a finding which throws further doubt on the likelihood that the phospholipids play a role in the production of milk fat.

Phospholipids.—During this year additional differences between the behavior of lecithin and cephalin have been recorded. Chargaff & Ziff (67) find that whereas cephalin, the more acidic of the two, forms insoluble compounds with thymus histone between pH 2 and 7, lecithin

reacts in such a manner only between pH 7 and 8. The latter gives no precipitate with the globin of the thymus whereas the former definitely does so at a pH below 4. The ability of the histone to inhibit blood coagulation is ascribed to the precipitation of cephalin. This phospholipid, in contrast to lecithin, splits the linkage between the prosthetic group and the protein in oxy- and carboxyhemoglobin (68). Christensen & Hastings (69) find that neither lecithin, cephalin, nor sphingomyelin combines with any appreciable quantities of the chloride ion and that lecithin and sphingomyelin do not combine with the sodium ion over a wide range of hydrogen ion concentration. Cephalin, however, binds sodium and potassium with equal affinity, the amount bound increasing with increase of pH. Lecithin and sphingomyelin show no buffering action over the pH range of 3 to 11. The conclusion is made that cephalin probably does not exist in aqueous solution as a zwitter ion, as has been proposed, and the suggestion is made that cephalin has a structure other than the one usually given. In this connection it should be recalled that the elementary analysis of purified cephalins has not agreed with the theoretical values (70). Weissberger's (71) observation that ammonium chloride acidosis causes an increase in the phospholipid turnover in the kidney led him to propose that these lipids are concerned with the maintenance of the acid-base equilibrium.

The question of the structure of other lipids is becoming more complicated. Thannhauser & Reichel (72) believe that the sphingomyelin isolated by them from a normal spleen exists as a mixture of lignoceryl sphingomyelin and lignoceryl sphingomyelin fatty acid ester. This would imply the binding of both hydroxyl groups of sphingosine, the possibility of which is substantiated from additional evidence presented by these workers (73). The fatty acids in the sphingomyelin-cerebroside fraction of the lipids of milk, like those in sphingomyelins and cerebrosides obtained from other sources, consist chiefly of lignoceric acid (74). The cerebroside isolated from the spleen in a case of Gaucher's disease differs from the usual ones in that it does not contain galactose but a fermentable sugar which was proven to be glucose (75). Klenk (76) found a new type of glycolipid in various lipoidoses. It is characterized by a content of sugar which far exceeds that found in normal cerebrosides. In contrast to the lipids from the brain in Niemann-Pick's disease, those isolated from the brain in a case of infantile amaurotic idiocy contained less sphingomyelin and much more of this new type of cerebroside.

Bloor (77) has continued his studies on the relationship between tissue activity and phospholipid content, and finds that the muscles of rats which had been exercised through the second and third generations contain larger amounts of phospholipids and cholesterol than those of unexercised animals.

Some conflicting observations have been recorded on the rate of phospholipid turnover. Sinclair (78), employing elaidic acid as a marker, has observed that whereas the transformation in the liver may be almost completed within one day, the reaction in the kidney is considerably slower, being only 60 per cent at the end of three days. Perlman *et al.* (79), on the other hand, using radioactive phosporus as a marker, had previously reported that the peak is reached in five to ten hours in the liver and within twenty-four hours in the kidney. Chargaff, Olson & Partington (80), also using radioactive phosphorus, confirm earlier observations that the turnover of lecithin in the liver and small intestine considerably exceeds that of cephalin but add that in partially hepatectomized animals (rats) the difference between the two almost disappears. Sinclair (81), however, finds no such difference when elaidic acid is employed and is not in accord with the idea that cephalin is entirely nonmetabolic in nature. These discrepancies resolve themselves into the question as to which indicator of metabolic activity is telling the truer story. Does the mere introduction of radioactive phosphorus indicate, of necessity, that the whole phospholipid molecule has been formed anew? Are there one or two elaidyl radicals present in these newly formed products? It would be helpful if these pertinent questions could be answered. The problem has not been clarified by the disconcerting observation that more of the radioactive phosphorus in dietary aminoethyl phosphoric acid was subsequently found in the lecithin portion of the liver and intestine of rats than in the cephalin fraction (82).

Chaikoff *et al.* (83) find that the phospholipid turnover of all parts of the central nervous system is at its maximum at birth. This is followed by a precipitous drop until the animals reach a weight of 50 gm., after which the turnover continues to decrease more slowly. It is most intense in the spinal cord from birth to the 50 gm. stage, after which the activities of the forebrain, cerebellum, and medulla rise; at a body weight of 200 gm. the turnovers are equal to or exceed that in the cord. At this time all are sluggish.

The testicular phospholipids are very stable and are not influenced by diet (84).

Cholesterol.—The value of the determination of blood cholesterol partition in surgery of the gall bladder has been discussed (85) and the use of intramuscular injection of cholesterol in various anemias of pregnancy has been proposed (86). The feeding of cholesterol in amounts sufficient to markedly increase the blood cholesterol (500 to 1000 mg. per cent) did not stimulate the development of tumors in rabbits exposed to ultraviolet light (87). High dietary cholesterol was found to have an inhibitory effect on the growth of rats, guinea pigs, and rabbits. The spleens and livers of guinea pigs and rabbits were enlarged, but in rats the livers only were affected (88). On the other hand, the intramuscular administration of cholesterol oleate into goats resulted in a slight increase in body weight and in the total lipid and cholesterol content of the milk (89). Dirscherl & Traut (90) failed to obtain any indication of a synthesis, in rats, of cholesterol from dietary fatty acids. Their experimental procedure is, however, open to the criticism that the amounts of acids fed were comparatively small. New analytical procedures which are merely modifications of older methods have been proposed (91, 92, 93).

Miscellaneous.—Wells (94) deplores the prevalence of the view that adipose tissue is merely connective tissue in which fat has been deposited, and gives reasons for his belief that fat tissue is a specific tissue, in fact a definite organ, which should be looked upon as a part of the reticulo-endothelial system. It is believed that tumors produced experimentally do not oxidize fatty acids by β-oxidation but derive energy as a result of ω-oxidation (95). In confirmation of previous work the yields of milk and milk fat in cows and goats increased after the administration of thyroxine. This was accompanied by a rise in blood sugar and a fall in blood lipids (96). The increase in milk production was ascribed to an increase in metabolic rate and food consumption as well as to a faster circulation of the blood. There was no evidence of heart damage (97).

Crystalline carotene is utilized by the growing chick even if the dietary fat is reduced to 0.1 per cent (98). During the onset of intensive feeding on a high fat diet the vitamin-A content of the liver of the cape salmon decreases (99). This is believed to show that vitamin A is associated with fat assimilation.

A micromethod for determining lecithin, cephalin, and sphingomyelin in small amounts of blood and tissue has been described (100). Data on blood plasma, cells, stroma, and brain are included. A new conversion factor for calculating β-hydroxybutyric acid from the

weight of the mercury precipitate has been proposed (101). Applications of recently developed analytical procedures to studies on lipid metabolism have been discussed (102).

Hilditch's recent book (103) contains many valuable data on the component fatty acids of numerous natural plant and animal fats. His description of the experimental technique for the quantitative investigation of fats is likewise very useful.

LITERATURE CITED

1. FRAZER, A. C., *Physiol. Rev.,* **20,** 561 (1940)
2. FREEMAN, L. W., AND JOHNSON, V., *Am. J. Physiol.,* **130,** 723 (1940)
3. MACHEBOEUF, M. A., AND PERRIMOND-TROUCHET, R., *Compt. rend. soc. biol.,* **132,** 274 (1939); *Chem. Abstracts,* **34,** 1691 (1940)
4. BELLINI, L., AND CERA, B., *Pathologica,* **32,** 195 (1940); *Chem. Abstracts,* **34,** 7350 (1940)
5. REISER, R., *J. Biol. Chem.,* **135,** 303 (1940)
6. WEIL, L., AND RUSSEL, M. A., *J. Biol. Chem.,* **136,** 9 (1940)
7. DEUEL, JR., H. J., HALLMAN, L. F., AND LEONARD, A., *J. Nutrition,* **20,** 215 (1940)
8. DEUEL, JR., H. J., AND HALLMAN, L. F., *J. Nutrition,* **20,** 227 (1940)
9. SPERRY, W. M., WAELSCH, H., AND STOYANOFF, V. A., *J. Biol. Chem.,* **135,** 281 (1940)
10. WAELSCH, H., SPERRY, W. M., AND STOYANOFF, V. A., *J. Biol. Chem.,* **135,** 291 (1940)
11. WAELSCH, H., SPERRY, W. M., AND STOYANOFF, V. A., *J. Biol. Chem.,* **135,** 297 (1940)
12. WAELSCH, H., AND SPERRY, W. M., *J. Biol. Chem.,* **132,** 787 (1940)
13. STETTEN, D., AND SCHOENHEIMER, R. S., *J. Biol. Chem.,* **133,** 329 (1940)
14. STETTEN, D., AND SCHOENHEIMER, R. S., *J. Biol. Chem.,* **133,** 347 (1940)
15. LANG, K., *Z. physiol. Chem.,* **261,** 240 (1939); *Chem. Abstracts,* **34,** 1337 (1940)
16. LANG, K., AND MAYER, H., *Z. physiol. Chem.,* **261,** 249 (1939); *Chem. Abstracts,* **34,** 1338 (1940)
17. LANG, K., AND MAYER, H., *Z. physiol. Chem.,* **262,** 120 (1939); *Chem. Abstracts,* **34,** 2400 (1940)
18. BERNHARD, K., AND SCHOENHEIMER, R., *J. Biol. Chem.,* **133,** 707 (1940)
19. BERNHARD, K., AND SCHOENHEIMER, R., *J. Biol. Chem.,* **133,** 713 (1940)
20. HILDITCH, T. P., AND PEDELTY, W. H., *Biochem. J.,* **34,** 40 (1940)
21. HILDITCH, T. P., AND PEDELTY, W. H., *Biochem. J.,* **34,** 971 (1940)
22. HOSTETLER, E. H., HALVERSON, J. O., AND SHERWOOD, F. W., *N. Carolina Agr. Expt. Sta. Tech. Bull.,* 61 (1939); *Chem. Abstracts,* **34,** 2485 (1940)
23. HUME, E. M., NUNN, L. C. A., MACLEAN, I. S., AND SMITH, H. H., *Biochem. J.,* **34,** 879 (1940)
24. BURR, G. O., BROWN, J. B., KASS, J. P., AND LUNDBERG, W. O., *Proc. Soc. Exptl. Biol. Med.,* **44,** 242 (1940)

25. HEVESY, G., AND SMEDLEY-MACLEAN, I., *Biochem. J.*, **34**, 903 (1940)
26. SCHNEIDER, H. A., STEENBOCK, H., AND PLATZ, B. R., *J. Biol. Chem.*, **132**, 539 (1940)
27. WICK, A. N., AND MACKAY, E. M., *Am. J. Physiol.*, **130**, 332 (1940)
28. DEUEL, JR., H. J., HALLMAN, L. F., GREELEY, P. O., BUTTS, J. S., AND HALLIDAY, N., *J. Biol. Chem.*, **133**, 173 (1940)
29. CONTE-MAROTTA, R., *Arch. sci. biol.*, **24**, 396 (1938); *Chem. Abstracts*, **34**, 166 (1940)
30. HARRISON, L. C., AND LONG, C. N. H., *J. Biol. Chem.*, **133**, 209 (1940)
31. JOHNSTON, C. G., IRVIN, J. L., AND WALTON, C., *J. Biol. Chem.*, **131**, 425 (1940)
32. JONES, K. K., AND SHERBERG, R. O., *Proc. Soc. Exptl. Biol. Med.*, **35**, 535 (1937)
33. BARRETT, H. M., BEST, C. H., MACLEAN, D. L., AND RIDOUT, J. H., *J. Physiol.*, **97**, 103 (1939)
34. ENTENMAN, C., MONTGOMERY, M. L., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **135**, 329 (1940)
35. CEDRANGOLO, F., AND BACCARI, V., *Arch. sci. biol. (Italy)*, **24**, 311 (1938); *Chem. Abstracts*, **34**, 166 (1940)
36. KILBORN, L. G., *Chinese J. Physiol.*, **14**, 283 (1939)
37. DRAGSTEDT, L. R., *J. Am. Med. Assoc.*, **114**, 29 (1940)
38. DRAGSTEDT, L. R., VERMEULEN, C., GOODPASTURE, W. C., DONOVAN, P. B., AND GEER, W. A., *Arch. Internal Med.*, **64**, 1017 (1939)
39. MONTGOMERY, M. L., ENTENMAN, C., GIBBS, G. E., AND CHAIKOFF, I. L., *Proc. Soc. Exptl. Biol. Med.*, **43**, 349 (1940)
40. LEITES, S. M., AND LUSENKO, V. S., *Bull. biol. méd. exptl. U.R.S.S.*, **8**, 342 (1939); *Chem. Abstracts*, **34**, 5135 (1940)
41. MCHENRY, E. W., AND GAVIN, G., *Science*, **91**, 171 (1940)
42. ENGEL, R. W., AND PHILLIPS, P. H., *J. Nutrition*, **18**, 329 (1939)
43. LONGENECKER, H. E., GAVIN, G., AND MCHENRY, E. W., *J. Biol. Chem.*, **134**, 693 (1940)
44. PERLMAN, I., STILLMAN, N., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **133**, 651 (1940)
45. PERLMAN, I., STILLMAN, N., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **135**, 359 (1940)
46. SINGAL, S. A., *J. Biol. Chem.*, **133**, xc (1940)
47. CHANNON, H. J., MANIFOLD, M. C., AND PLATT, A. P., *Biochem. J.*, **34**, 866 (1940)
48. CARTER, H. E., AND MELVILLE, D. B., *J. Biol. Chem.*, **133**, 109 (1940)
49. BEST, C. H., AND RIDOUT, J. H., *J. Physiol.*, **97**, 489 (1940)
50. BEESTON, A. W., AND PLATT, A. P., *J. Soc. Chem. Ind.*, **58**, 557 (1939)
51. SINGAL, S. A., AND ECKSTEIN, H. C., *Proc. Soc. Exptl. Biol. Med.*, **41**, 512 (1939)
52. TUCKER, H. F., AND ECKSTEIN, H. C., *J. Biol. Chem.*, **121**, 479 (1937)
53. TUCKER, H. F., AND ECKSTEIN, H. C., *J. Biol. Chem.*, **126**, 117 (1938)
54. TUCKER, H. F., TREADWELL, C. R., AND ECKSTEIN, H. C., *J. Biol. Chem.*, **135**, 85 (1940)

55. ENTENMAN, C., LORENZ, F. W., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **133**, 231 (1940)
56. BÜRGER, M., *Z. ges. Neurol. Psychiat.*, **167**, 273 (1939); *Chem. Abstracts,* **34**, 1721 (1940)
57. BARKER, N. W., *Ann. Internal Med.*, **13**, 685 (1939)
58. STROEBE, F., AND THADDEA, S., *Z. klin. Med.*, **135**, 612 (1939); *Chem. Abstracts,* **34**, 6346 (1940)
59. GILDEA, E. F., MANN, E. B., AND PETERS, J. P., *J. Clin. Investigation,* **18**, 739 (1939)
60. MANN, E. B., GILDEA, E. F., AND PETERS, J. P., *J. Clin. Investigation,* **19**, 43 (1940)
61. FLEISCHMANN, W., SHUMACKER, H. B., AND WILKINS, L., *Am. J. Physiol.,* **131**, 317 (1940)
62. RUBIN, S. H., *J. Biol. Chem.,* **131**, 691 (1939)
63. ARTOM, C., AND FREEMAN, J. A., *J. Biol. Chem.,* **135**, 59 (1940)
64. ERICKSON, B. N., SOUDERS, H. J., SHEPHERD, M. L., TEAGUE, D. M., AND WILLIAMS, H. H., *Proc. Soc. Exptl. Biol. Med.,* **45**, 153 (1940)
65. ENTENMAN, C., CHANGUS, G. W., GIBBS, G. E., AND CHAIKOFF, I. L., *J. Biol. Chem.,* **134**, 59 (1940)
66. VORIS, L., ELLIS, G., AND MAYNARD, L. A., *J. Biol. Chem.,* **133**, 491 (1940)
67. CHARGAFF, E., AND ZIFF, M., *J. Biol. Chem.,* **131**, 25 (1939)
68. CHARGAFF, E., ZIFF, M., AND HOGG, B. M., *J. Biol. Chem.,* **131**, 35 (1939)
69. CHRISTENSEN, H. N., AND HASTINGS, A. B., *J. Biol. Chem.,* **136**, 387 (1940)
70. GRAY, E. L., *J. Biol. Chem.,* **136**, 167 (1940)
71. WEISSBERGER, L. H., *J. Biol. Chem.,* **132**, 219 (1940)
72. THANNHAUSER, S. J., AND REICHEL, M., *J. Biol. Chem.,* **135**, 1 (1940)
73. REICHEL, M., AND THANNHAUSER, S. J., *J. Biol. Chem.,* **135**, 15 (1940)
74. KURTZ, F. E., AND HOLM, G. E., *J. Dairy Sci.,* **22**, 1011 (1939); *Chem. Abstracts,* **34**, 1086 (1940)
75. HALLIDAY, N., DEUEL, H. J., TRAGERMAN, L. J., AND WARD, W. E., *J. Biol. Chem.,* **132**, 171 (1940)
76. KLENK, E., *Z. physiol. Chem.,* **262**, 128 (1939); *Chem. Abstracts,* **34**, 2060 (1940)
77. BLOOR, W. R., *J. Biol. Chem.,* **132**, 77 (1940)
78. SINCLAIR, R. G., *J. Biol. Chem.,* **134**, 71 (1940)
79. PERLMAN, I., RUBEN, S., AND CHAIKOFF, I. L., *J. Biol. Chem.,* **122**, 169 (1937–38)
80. CHARGAFF, E., OLSON, K. B., AND PARTINGTON, P. E., *J. Biol. Chem.,* **134**, 505 (1940)
81. SINCLAIR, R. G., *J. Biol. Chem.,* **134**, 83 (1940)
82. CHARGAFF, E., AND KESTON, A. S., *J. Biol. Chem.,* **134**, 515 (1940)
83. FRIES, B. A., CHANGUS, G. W., AND CHAIKOFF, I. L., *J. Biol. Chem.,* **132**, 23 (1940)
84. SINCLAIR, R. G., *J. Biol. Chem.,* **134**, 89 (1940)
85. PICKHARDT, O. C., BERNHARD, A., AND KOHN, I. L., *Ann. Surg.,* **110**, 701 (1939)
86. CHATTERJEE, H. N., *Lancet,* **I**, 14 (1940).

87. BAUMANN, C. A., RUSH, H. P., KLINE, B. E., AND JACOBI, H. P., *Am. J. Cancer,* **38,** 76 (1940)

88. COOK, R. P., AND McCULLAGH, G. P., *Quart. J. Exptl. Physiol.,* **29,** 283 (1939)

89. TORRISI, D., *Arch. fisiol.,* **39,** 431 (1940); *Chem. Abstracts,* **34,** 4128 (1940)

90. DIRSCHERL, W., AND TRAUT, H., *Z. physiol. Chem.,* **262,** 61 (1939); *Chem. Abstracts,* **34,** 1368 (1940)

91. KAYE, I. A., *J. Lab. Clin. Med.,* **25,** 996 (1940)

92. ALBERT, H. K., AND BRYANT, J. T., *J. Franklin Inst.,* **229,** 118 (1940)

93. HOFFMAN, W. S., *Proc. Soc. Exptl. Biol. Med.,* **43,** 38 (1940)

94. WELLS, H. G., *J. Am. Med. Assoc.,* **114,** 2177, 2284 (1940)

95. CIARANFI, E., *Arch. sci. biol. (Italy),* **26,** 8 (1940); *Chem. Abstracts,* **34,** 8039 (1940)

96. SMITH, J. A. B., AND DASTUR, N. N., *Biochem. J.,* **34,** 1093 (1940)

97. RALSTON, N. P., COWSERT, W. C., RAGSDALE, A. C., HERMAN, H. A., AND TURNER, W. C., *Missouri Agr. Expt. Sta. Research Bull.,* 317 (1940); *Chem. Abstracts,* **34,** 6984 (1940)

98. RUSSEL, W. C., TAYLOR, M. W., AND POLSKIN, L. J., *J. Nutrition,* **19,** 555 (1940)

99. MOLTENO, C. J., AND RAPSON, W. S., *Biochem. J.,* **33,** 1390 (1939)

100. ERICKSON, B. N., AVRIN, I., TEAGUE, D. M., AND WILLIAMS, H. H., *J. Biol. Chem.,* **135,** 671 (1940)

101. BLUNDEN, H., HALLMAN, L. F., MOREHOUSE, M. G., AND DEUEL, H. J., *J. Biol. Chem.,* **135,** 757 (1940)

102. ECKSTEIN, H. C., *J. Lab. Clin. Med.,* **26,** 184 (1940)

103. HILDITCH, T. P., *The Chemical Constitution of Natural Fats* (John Wiley and Sons, Inc., New York, 1940)

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THE METABOLISM OF PROTEINS AND AMINO ACIDS

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This review does not pretend to offer a complete list of all papers on amino acid and protein metabolism, but rather to discuss certain selected topics to which, in the opinion of the reviewers, important contributions have been made. Difficulties in consulting recent European literature may have resulted in unintentional omissions. As this volume includes separate chapters on the metabolism of sulfur and on detoxification, only brief reference has been made to those papers, the main subject of which is considered in other chapters. The interaction of three amino acids in the biological synthesis of creatine, established during the review period, requires a short discussion in this chapter. As the application of isotopes to the study of metabolic processes represents only one of the many methods available, no separate section is included.

General Aspects of Protein Metabolism

Madden & Whipple (1) have reviewed the results of experiments on regeneration of plasma proteins, which have an important bearing on the general concept of protein metabolism. When blood plasma proteins are depleted by bleeding, with return of the washed red cells (plasmapheresis), it is possible to bring dogs on a low protein diet to a steady state of hypoproteinemia and a uniform plasma protein formation. The dog has in the organ proteins a definite protein reserve (10 to 60 gm.) which may be utilized as plasma protein building material. The complete removal of this reserve material ("reserve store") requires about two to six weeks of plasmapheresis (2). The reserve stores can be replenished and the animals kept in nitrogen equilibrium not only by the administration of an adequate diet but also by the intravenous injection of homologous plasma (3). These results in conjunction with earlier ones are interpreted as an indication of a reversible flow of plasma proteins to organ proteins, and demonstrate the "fluidity of the body protein (including plasma protein)—a ready give and take between the protein depots—a dynamic equilibrium of body protein."

Experiments in which the fate of dietary amino acids in normal

animals was studied with isotopes have given rise to a similar concept of the high metabolic activity of the body proteins. The results of the isotope work have been recently reviewed (4). An experiment with glycine containing N^{15} (5) was carried out under the same conditions as earlier ones in which $l(-)$-leucine and dl-tyrosine had been investigated. About half of the glycine nitrogen entered into the various body proteins, and only 40 per cent was recovered in the urine. Only a relatively small amount of the isotope in the protein was located in the glycine, and most of the remainder was found in other amino acids. The isotope in the arginine was located in its amidine group, indicating that this protein constituent had lost its original amidine group (probably as urea) and had accepted a new one. The general results are thus almost the same as those reported after administration of the other amino acids, and seem to indicate the general course taken by most, if not all, dietary amino acids. As the total amount and constitution of the tissue proteins of the experimental animals must have remained constant within narrow limits, the various chemical reactions of the tissue proteins must have been so balanced as to avoid changes. Most of these chemical processes require rapid intermittent rupture of the protein molecule, i.e., opening and closing of peptide linkages as well as coupled deamination and amination of liberated amino acids. In addition to the rapid nitrogen transfer there occurs in normal animals also a continuous conversion of the carbon chain of some amino acids into others. Such conversions as that of phenylalanine into tyrosine (6) and of ornithine into proline, glutamic acid, and arginine (7, 8), discussed later, are automatic reactions which go on even if the reaction products (i.e., tyrosine, glutamic acid, etc.) are abundantly supplied in the diet. As the compounds newly formed by interconversion also enter protein linkage in the same manner as those entering the body with the diet, they furnish another indication of the biological lability of peptide linkages in proteins.

Preliminary experiments with N^{15} seem to indicate that the proteins of plants are involved in automatic reactions similar to those of animals. Tobacco plants, when kept on a culture medium containing isotopic ammonia, yielded proteins with an isotope concentration higher than could be accounted for by the limited growth of the plants. Amino acids (glutamic acid, histidine, and arginine) isolated from the proteins of the leaves contained relatively high amounts of N^{15} (9). The uptake of isotopic nitrogen from the culture solution by the pro-

tein of sunflowers was investigated with similar results by Hevesy and associates (10). These results in conjunction with those obtained with animals tend to show that the rapid and continuous chemical regeneration of the cell proteins is a general characteristic of living matter.

Burroughs, Burroughs & Mitchell (11) have carried out new experiments on the independence of the "exogenous" and "endogenous" nitrogen metabolism, the latter being measured by the nitrogen excretion of rats on a nitrogen-free diet. The addition of small amounts of amino acid mixtures or even of proteins of high biological value (egg) did not change the "endogenous" quota. They uphold the classical theory of Folin of the two independent quotas of nitrogen metabolism, a concept scarcely to be reconciled with the recent results of isotope work, which have revealed the rapid metabolic merging of tissue and dietary components. The results of the isotope technique are given a new intepretation by these authors: The chemical activity of the body proteins revealed by the isotope method is restricted to the "dispensable reserve proteins," which are supposed to be the same part of the tissue protein as was formulated by Whipple and his associates (1) to function as a reserve store for plasma protein formation. The "fixed" proteins, however, are supposed to be stationary and not involved in the reactions observed with isotopes. According to this new theory one would have to distinguish between the catabolism of food and dispensable reserve proteins on the one hand and that of specific cell constituents on the other.

The discrepancy between the different views might not be so great, in the opinion of the reviewers, if the reactivity of the various proteins be considered from the quantitative, rather than the qualitative point of view. While all organ proteins are involved, they react at widely different rates.

Schmidt and associates (12) have pointed out the theoretical possibility that proteins may be interconverted without cleavage of peptide linkages, i.e., by chemical reactions affecting only the free side groups of protein-bound amino acids. By the introduction, for instance, of a hydroxyl into the phenyl group of combined phenylalanine, the phenylalanine and tyrosine contents would change, resulting in the formation of a protein with new properties.

Dirr (13) reported that the administration of $l(+)$-arginine results in an increase of arginine in the serum proteins. From this and analogous results with histidine (14) and tyrosine (15), one might

be tempted to conclude that the chemical composition of the serum protein is a function of the amino acid content of the diet. However, the effect with arginine could not be corroborated by Block (16). The experiments with tyrosine and histidine thus also require repetition.

The amino acid composition of urinary proteins obtained from a nephritic patient was compared with that of normal and nephritic serum (17). The urinary protein composition was independent of the dietary regime. It does not represent total serum protein, but rather serum albumin or a mixture consisting mainly of albumin with some globulin.

Individual Amino Acids

Glycine.—The indispensability of glycine in the diet of fowls (18, 19) is discussed on page 203. The contradictory reports concerning glycogenesis from glycine now appear to be reconciled by the observation of MacKay, Wick & Carne (20) that it forms glycogen to the same extent as alanine but at a slower rate. The expected antiketogenic activity becomes apparent when ketone bodies in blood and urine are determined (21).

The rapid metabolic demethylation of sarcosine first demonstrated by Abbott & Lewis (22) has been confirmed by the use of isotopic nitrogen. The liberation of glycine is so rapid that the feeding of isotopic sarcosine has almost the same effects as that of isotopic glycine (23).

Hippuric acid synthesis has been studied with tissue slices by Borsook & Dubnoff (24). As the formation (i.e., peptide synthesis) is associated with a gain of free energy, it must thus be coupled *in vivo* with another energy-liberating reaction. The process can be blocked by poisoning cellular respiration.

Phenylalanine and tyrosine.—The direct conversion of phenylalanine into tyrosine has been demonstrated by feeding a phenylalanine preparation with stably bound deuterium located in the phenyl ring (6). Tyrosine samples isolated from the proteins of liver or other body tissues contained a high concentration of deuterium. The conversion and the introduction of the newly formed tyrosine into the proteins must be very rapid processes. The oxidation of phenylalanine to tyrosine proceeds even when considerable amounts of nonisotopic tyrosine are also incorporated in the diet.

The studies on the relation of ascorbic acid to the metabolism of the phenylated amino acids have been extended by Sealock & Silber-

stein (25, 26). The excretion of homogentisic acid, p-hydroxyphenyl-pyruvic acid, and p-hydroxyphenyllactic acid by guinea pigs on a diet deficient in vitamin C occurs after feeding either tyrosine or phenylalanine but ceases when definite doses of ascorbic acid are administered. The action of the vitamin is specific, as isoascorbic acid is effective only in relation to its antiscorbutic activity. Hereditary human alcaptonuria, however, is resistant to ascorbic acid medication.

Block and his associates have reported that administration of phenylpyruvic or phenyllactic acid to patients with phenylpyruvic oligophrenia, increases the content of free phenylalanine in the blood (27), indicating that the patients are able to aminate these phenyl-alanine derivatives. The excretion of phenylpyruvic acid is therefore probably due only to deamination of phenylalanine in the kidney, since the results seem to indicate that the metabolic disturbance lies in a curtailed ability to metabolize phenylalanine. Proteins from the blood and tissues of such patients have the same amino acid composition as do normal individuals (28).

Dicarboxylic acids.—α-Ketoglutaric acid, which is known to be easily converted into glutamic acid *in vivo,* can be synthesized by minced pigeon liver from pyruvic acid and carbon dioxide. This reaction, established with the aid of radioactive bicarbonate by Evans & Slotin (29), demonstrates that carbon dioxide must be regarded as one of the building materials of glutamic acid.

A new mechanism for the oxidation of $l(+)$-glutamic and $l(+)$-aspartic acids has been offered by Felix & Naka (30) on the basis of experiments with tissue slices from rat organs. The compounds were found to be highly resistant to liver slices but to be rapidly oxidized by kidney tissue. Glutamic acid took up two mols, and aspartic acid one mol of oxygen with liberation of two and one mols of carbon dioxide respectively. Only one third of the nitrogen was liberated as ammonia and no keto acids were formed. These results are in contrast to those of Krebs (31), who isolated the corresponding α-keto acids when tissues of dogs, rabbits, and guinea pigs were employed. The discrepancies may thus be due to a difference of animal species. The author suggests that the oxidation of glutamic acid by rat kidney starts at the ω-carbon atom and yields a three-carbon chain without loss of nitrogen. Glutamic and aspartic acids may yield the same unknown compound.

By employing more specific methods for the determination of glutamic and aspartic acids, and using a purified enzyme from either

pigeon breast or pig heart muscle, Cohen (32) has extended his work which showed that transamination involves only a few amino acids. This reversible reaction seems to be restricted mainly to glutamic acid, aspartic acid, alanine, and their corresponding keto acids. The finding of Braunstein (33) that cysteic acid can replace the natural dicarboxylic acids is corroborated, but no evidence was found for the existence of two separate enzymes, one active only on glutamic and the other on aspartic acid. The term transaminase is now proposed for the enzyme. Only amino acids of the *l*-series are active with transaminase, but glutathione is not. A study of the kinetics of transaminase activity is described in a separate publication (34).

Basic amino acids.—$l(+)$-Lysine, in contrast to most other amino acids, is not deaminized by kidney or liver slices (30).

The conversion of ornithine into other amino acids was investigated with deuterium. Deuterioornithine was synthesized via deuteriopiperidone and administered to adult mice in addition to their ordinary casein-containing stock diet. Arginine (8), proline, and glutamic acid (7) isolated from the proteins of the animals contained amounts of isotope indicative of their origin from the ornithine. As the diet contains large amounts of glutamic acid and considerable quantities of proline and arginine, the conversion, like that of phenylalanine to tyrosine, is an automatic reaction. The formation of isotopic arginine, in conjunction with evidence of the replacement of its amidine group, is indication that the arginine of protein is involved in the ornithine cycle during the frequent intervals when it is in the free state (4).

The conversion of ornithine and citrulline into arginine, and the function of the latter as a precursor of creatine, is discussed on pages 207 and 212.

The loss of nutritive value of heat- or alcohol-treated proteins may be due to the formation of new, nondigestible linkages between the amino groups of lysine and those of free carboxyl groups (35). Hydrolysis of such proteins therefore restores their biological value.

Histidine has again been shown (36, 37) to be a normal constituent of urine, which is excreted in widely varying amounts. It cannot be taken as a criterion of pregnancy (38).

Sulfur-containing amino acids.—The mode of inheritance of canine cystinuria has been studied further (39, 40) and a new case, also occurring in an Irish terrier, has been reported (41).

The growth-retarding effect of some carcinogenic hydrocarbons

(methyl cholanthrene, benzopyrene, and pyrene) was found by White & White to be alleviated by the administration of *l*-cystine, *dl*-methionine, *l*-cystine disulfoxide, or glutathione, whereas taurine, sodium sulfate, and glycine were ineffective (42). The toxic action is probably due to an increased requirement for the organic sulfur necessary for detoxification. The feeding of S-benzyl glutathione gives rise to the excretion of N-acetyl-S-benzyl-*l*-cysteine (43), indicating that the peptide linkage is split before acetylation. *dl*-Methionine sulfoxide supports the growth of rats on a diet low in methionine (44).

Indispensable Amino Acids

The amino acid requirements of chicks are somewhat different from those of rats. Glycine has now been shown by Almquist and associates to be one of the components of the essential growth factor present in polished rice but absent in yeast (18, 45). This amino acid is thus essential for promoting growth of chicks. The feeding of glycine results in an increase of muscle creatine, and in fact this amino acid can be replaced by creatine in the diet (19). This indicates that it "is required for the synthesis of creatine and that in a deficiency of glycine, creatine synthesis is retarded."

Earlier work has shown that arginine is also essential for maintenance and growth of the chick (46). Ornithine or urea, or both of these together, cannot replace arginine, indicating that the chick lacks the mechanism for arginine synthesis present in mammals (47). As citrulline, however, may serve instead of arginine (48), the chick has the ability of substituting an imino group for the oxygen of the ureido group of citrulline.

Burroughs, Burroughs & Mitchell (49) (see also page 199) distinguish between amino acids essential for growth as defined by Rose (50) and those essential for maintenance of nitrogen equilibrium on a low nitrogen diet. The nitrogen was supplied in the form of amino acid mixtures. If the removal of one or more amino acids from this mixture resulted in a negative nitrogen balance, such acids were considered to be "essential." The only amino acids found to be necessary for the maintenance of nitrogen equilibrium under these conditions are threonine, isoleucine, norleucine, tryptophane, valine, methionine, and phenylalanine or tyrosine. A certain interdependence appears to exist between the essential amino acids, for threonine and isoleucine, either individually or together, are necessary for the utilization of the others. The other amino acids known to be indispensable for growth are not

considered essential for maintenance of nitrogen equilibrium. About 30 to 50 per cent of the nitrogen may be supplied by nonspecific amino acid nitrogen sources. The assumption is made in this interpretation, which requires the distinction between exogenous and endogenous metabolism, that utilization of nitrogen in the adult animal is only for the replacement of endogenous losses. The authors consider that only in growth does total protein synthesis occur, and for this all essentials must be present, while in the adult animal only "particular requirements" must be met for maintenance of nitrogenous integrity.

The effect of individual amino acids on the plasma protein production of dogs with experimental hypoproteinemia (see also page 197) was investigated by supplementing the diet with various amino acid mixtures (3). Under these experimental conditions, cystine, leucine, and glutamic acid appear to be of primary importance in the building of new plasma proteins, and of these cystine occupies a key position. The effect of individual amino acids on hemoglobin formation was studied in a similar way by amino acid supplements to the basal ration of standard anemic dogs (51). Almost all of the amino acids employed increase hemoglobin formation in varying degrees, and the racemic forms were generally as effective as the natural isomerides. The effect is also observed with isovaleric, β-hydroxybutyric, and glutaric acids.

d-Amino Acids

Tumor proteins.—The claims of Kögl and collaborators[1] to the effect that the proteins of cancer tissue are characterized by the presence of amino acids of unnatural configuration and especially of *d*-glutamic acid, have again received support from Arnow, Opsahl & Watson (52), who found partially racemized glutamic acid in a sarcoma of dog kidney.

Kögl, Erxleben & Akkerman (53) ascribe the many failures to corroborate their findings partly to the use of the Foreman method in isolation of glutamic acid, stating that the calcium (or barium) salt of the racemic mixture is considerably more soluble than that of the *d*- or *l*-component. This claim has been denied independently by Chibnall, Rees, Williams & Boyland (54) and by Graff, Rittenberg & Foster (55), who observed no difference in the solubility of the salts.

[1] *Ann. Rev. Biochem.,* **9,** 282 (1940).

dl-Glutamic acid added to a hydrolysate was recovered by this proce-
dure in both laboratories.

Various tumor types have been investigated with respect to their
amino acid content by Kögl & Erxleben (56), who have found
d-glutamic acid in cancer metastases from lung and spleen, and the
same substance, together with small amounts of racemized leucine and
valine, in a rabbit myxoma investigated as a virus type of tumor. The
amounts of *d*-amino acids in an osteosarcoma were similar to those
in cancer, but this tumor was distinguished by the presence of par-
tially racemized arginine, a finding which, if corroborated, might ac-
cording to the authors indicate a chemical difference between the two
malignant types. Uterine myoma, employed as a prototype of benign
growth, contained very small amounts of *d*-leucine, *d*-proline, and
d-hydroxyglutamic acid, but only the natural form of glutamic acid.
Only normal amino acids were found by Arnow, Opsahl & Watson
in liver and spleen of lymphatic leukemia (52), in embryonic tissue
(57), and by Schramm & Müller (58) in tobacco mosaic virus.

Kögl & Erxleben have investigated the action of proteolytic en-
zymes on peptides of unnatural configuration by feeding boiled tumor
tissues to normal dogs (59). Partially racemized glutamic acid was
isolated from the excreta, but only after hydrolysis, and was therefore
presumed to have been excreted in peptide form. The results were
presented as additional evidence for the presence of *d*-amino acid in
tumors.

Six different laboratories have reported their failure to differen-
tiate normal from tumor proteins by the *d*-glutamic acid content (54,
55, 60 to 63). Chibnall *et al.* (54, 64) have isolated glutamic acid in
large and almost quantitative yields from normal animals and plants as
well as from tumors. The isolated material was fractionally crystallized
and the final mother liquors yielded only small amounts of the racemic
mixture. Such traces, however, were found in all protein hydroly-
zates. Chibnall suggests that the results of Kögl, who had frequently
obtained small yields of total glutamic acid, are due to the preferen-
tial precipitation of the *dl*- form. If the amounts of *d*-glutamic acid
found by Kögl had been expressed as a fraction of the total glutamic
acid or total protein, the corresponding proportionate values would
have been equally small. Traces of *d*-glutamic acid in both normal and
tumor proteins may have originated during protein hydrolysis, as this
amino acid is slowly racemized when boiled with hydrochloric acid
(65, 66, 67).

Two new methods have been employed, also with negative results, for the detection of *d*-amino acids in tumors. When treated with the specific enzyme, *d*-amino acid oxidase, Lipmann, Behrens, Kabat & Burk (60) found that the hydrolyzates of tumor tissue gave the same results as ordinary hydrolyzates, while the addition of *d*-glutamic acid became apparent by the recovery of 75 per cent of its nitrogen as ammonia. In support of Kögl's theory Arnow & Opsahl (68) have criticized this method as being too insensitive, since the enzyme must also have liberated nitrogen from amino acids (serine, cystine, etc.) known to be racemized during hydrolysis. Such an explanation, however, as pointed out by Behrens, Lipmann, Cohn & Burk (67), requires not only the presence in the tumor proteins of *d*-glutamic acid, but also an equivalent decrease of those other amino acids that become partially racemized during hydrolysis.

The absence of significant amounts of *d*-glutamic acid in tumor proteins has furthermore been demonstrated by the isotope dilution method of Rittenberg & Foster (69), which had already been employed for the quantitative determination of individual amino acids and fatty acids in mixtures. Graff, Rittenberg & Foster (55) have added *dl*-glutamic acid containing N^{15} to hydrolyzates of tumor proteins, and samples of *l*- and *dl*-glutamic acid were subsequently isolated. The dilution of the isotopic nitrogen in both samples permits the determination of the total amounts of both *l*- and *d*-glutamic acid with high accuracy. While the amount of total glutamic acid nitrogen in the hydrolyzates was high (6.7 to 8.7 per cent) the content of *d*-glutamic acid fell within the limit of error of the method.

Steric inversion of amino acids.—The biological inversion of $d(+)$-leucine into its natural isomeride was studied with a synthetic preparation in which deuterium was present as a marker of the carbon chain and N^{15} as a marker of the amino group (70). $l(-)$-Leucine, isolated from the organ proteins of the animals given the isotopic compound, contained a considerable concentration of the carbon chain marker (deuterium) but practically no nitrogen isotope, indicating that during the inversion, the nitrogen originally present in the amino group was replaced by nitrogen from other amino acids. The inversion is thus carried out in two steps: (*a*) complete deamination of the *d*-amino acid probably to the keto acid, and (*b*) amination of the keto acid. Part of the N^{15} was recovered in other amino acids of the proteins, showing that the nitrogen of the unnatural leucine may be employed in other amination reactions.

d-Amino acid oxidase.—The rate of oxidation of *d*-amino acids with kidney extracts, according to Felix & Zorn (71), varies considerably. While the oxidation of many amino acids is rapid, that of *d*-leucine, *d*-glutamic acid, *d*-arginine, *d*-serine, and *d*-histidine is slow, and *d*-lysine and glycine are entirely resistant. Putrescine and cadaverine are oxidized to aldehydes.

There is evidence that the action of the crude preparations employed by most investigators is due to the presence of more than one enzyme. Karrer & Frank (72), working with an enzyme synthesized from lactoflavin-adeninenucleotide and the apoenzyme from sheep kidney, found that the *d*-forms of alanine, leucine, isoleucine, phenylalanine, methionine, and proline react while those of glutamic acid, aspartic acid, arginine, histidine, dihydroxyphenylalanine, serine, and several peptides are entirely resistant.

The lowered amino oxidase activity in the tissue of riboflavin-deficient animals is due to an actual riboflavin deficiency of the organs, and can be restored by supplementing the diet (73, 74, 75), or even by adding the nucleotide to the tissues *in vitro* (76). The specific protein component of the enzyme is thus present even in deficient organs. Another indication for the dependence of enzyme activity on the quality of the diet is the finding that the kidneys of carnivorous animals have the highest activity, those of omnivorous a lower one, and those of herbivorous the lowest (77).

Urea and Ammonia

Bach (78) has corroborated the observations of Leuthardt (79) according to which glutamine, ammonia, and carbon dioxide form urea even in the absence of ornithine, indicating that there exists a second mechanism of urea formation which is independent of the ornithine cycle. Bach suggests now the occurrence of a third mechanism: citrulline when treated with surviving liver slices may yield urea without conversion into arginine and ornithine. In the presence of α-keto acids, citrulline is oxidized directly to urea and glutamic acid. The evidence for this reaction is based mainly on the failure to observe an increase of amino nitrogen in the reaction mixture, such as would be expected if ornithine were formed.

Most evidence still points to the ornithine cycle as the principle mechanism of urea formation. A new indication for the close relationship between urea formation and arginase activity in the liver is given by Munro (80). During amphibian development a change from the

predominant excretion of ammonia to that of urea occurs, with a concomitant appearance and increase of liver arginase.

According to the theory of urea formation by the ornithine cycle with liver slices, the carbon of the urea is ultimately derived from the carbonate ion of the buffer. This assumption has now been proved independently in two laboratories by Evans & Slotin, and by Rittenberg & Waelsch, who employed the radioactive isotope C^{11} with a half lifetime of twenty-one minutes (81) and the stable isotope C^{13} (82) respectively. The modes of experimentation were almost identical. Urea formation was carried out by liver slices in the presence of ornithine, ammonia, glucose or pyruvate, and isotopic bicarbonate buffer. The procedures employed for isolation of the isotopic material differed only slightly; in the former case the product was isolated as the dixanthydryl derivative; in the latter, carbon dioxide was released by the action of urease. The carbon of the urea formed in both series of experiments contained a concentration of isotope about half that in the carbon of the bicarbonate, i.e., half of the carbon of the urea was derived directly from the buffer and the rest probably from carbonate formed by the oxidation of the metabolite.

Herrin (83) has studied the secretion of ammonia into the intestinal lumen. Urea or protein administration resulted in an increased amount of ammonia in the intestinal loop. The process was independent of the urea concentration in the blood. Ammonia secretion thus seems to be a function of the metabolism of the intestinal gland cells.

TRANSMETHYLATION

One of the most interesting discoveries of the last year is the occurrence of a metabolic transfer of methyl groups from methionine to choline and creatine. Such transmethylation had been proposed by du Vigneaud and associates (84) as an explanation of the results which showed that choline is indispensable when methionine is replaced by homocystine in the diet of growing rats. They suggested that "the methyl group attached to the sulfur of methionine may be the source of the methyl groups in choline."

The transfer of the methyl groups from methionine to creatine was demonstrated by Borsook & Dubnoff (85). Liver slices of cats, rabbits, and rats slowly converted guanidoacetic acid into creatine, which was measured with the specific enzyme of Dubos & Miller. The addition of methionine increased the rate and extent of this

reaction. This action of methionine seems to be highly specific, as none of the other thirty-two compounds tested (amino acids, choline, betaine, mono-, di-, tri- and tetramethyl ammonium salts) had the same effect.

The direct and unequivocal proof for the methyl-shift was given with the aid of isotopes. Du Vigneaud, Chandler, Cohn & Brown (86) have synthesized methionine in which the three hydrogen atoms of the S-methyl group were present in the form of deuterium, while the other part of the amino acid molecule did not contain the isotope. Seventy mg. of this substance were added daily to a diet free of methionine and choline, which was given to immature rats. Choline and creatine isolated from the bodies contained a concentration of deuterium indicating that at least 56 per cent of the methyl groups of the choline and 69 per cent of those of creatine were derived from the dietary methionine.[2] A value of 100 per cent could scarcely have been obtained in these experiments as the tissues of the animals at the start of the experiments contained nonisotopic methyl groups in their methionine, choline, and creatine, which must have "diluted" the isotopic contents of the products isolated. The relatively high values obtained make it questionable whether any other source for methyl groups exists in normal diet or tissue. The authors suggest that "the presence of methyl groups in a utilizable form such as methionine or choline, may be essential in the diet."

These experiments have thrown new light on the mechanism of choline deficiency. Griffith & Wade (87) have observed that choline deficiency, which results in fatty livers in adult rats, also causes severe hemorrhagic degeneration of the kidneys in young rats. The hemorrhages, which thus constitute another symptom of methyl deficiency, are prevented by choline, betaine, or methionine, and their extent is increased by the addition of cystine, lard, or cholesterol to the diet. These are the same substances that increase the amount of fat in the liver of older rats (88, 89, 90). The addition of creatine decreases the severity of hemorrhagic degeneration, but does not prevent it. The authors suggest that though the methyl group of creatine is probably not available for the synthesis of choline, the effect may be due to a sparing action. The addition of creatine makes

[2] Choline may also yield its methyl group for creatine formation, as the feeding of choline with deuterium in its methyl groups resulted in the excretion of deuteriocreatinine (86a).

available for choline synthesis those methyl groups which otherwise would be used in the synthesis of creatine (91).

The lipotropic action of methionine when added to a choline-free diet of growing rats (92) has been confirmed by Best & Ridout (93), and the "unnatural" isomer of methionine was found to have about the same effect as the "natural" form or as the racemic mixture.

The well-established lipotropic action of betaine is probably also due to its function as a methyl donor. While not quite as effective as choline, this compound, administered orally or parenterally, acts like choline when methionine is replaced by homocystine (94).

The antagonistic action of cystine on the one hand and of choline and methionine on the other has been studied further by Channon, Manifold & Platt (95). The cystine content of the dietary proteins, according to Tucker, Treadwell & Eckstein (96), plays an important role in regard to the action of methionine. The lipotropic action of various proteins depends not so much upon the methionine content as upon the ratio of the antagonistic methionine to cystine.

Some betaines other than that of glycine (i.e., betaines of threonine, serine, and allothreonine) (97) or S-methyl cysteine (95) have been found to exert no lipotropic action. These findings together with reports of the inactivity of other methylated compounds (sarcosine, etc.) seem to indicate that the methyl groups, in order to be available for transmethylation, have to be attached to specific molecular groupings.

The excretion of a volatile selenium compound after administration of sodium selenite, suggested by Hofmeister (98) to be dimethyl selenide, is not increased by feeding choline or methionine (99). This might suggest that the formation of this methylated compound is not related to the physiological transmethylation. However, the identity of the volatile selenium compound is not yet definitely established.

Amino Acids as Precursors of Creatine

The immediate metabolic precursors of all parts of the creatine (and creatinine) molecule have been established during the period of this review.

The greatest difficulty in the study of creatine formation has always been the relative independence from the diet of the total amount of creatine and creatinine in tissues and urine. Balance experiments by feeding particular nitrogenous compounds to animals

or humans have never yielded definite indications in regard to the precursors, as creatine formation cannot be increased above the physiological needs of the cell.[3] The experiments of Hyde (100), who fed glycine and histidine to normal humans without resultant extra creatine or creatinine excretion, give additional support to this rule. The evidence for creatine precursors up to last year seems to have rested mainly on the experiments of Brand and his associates (101), who showed that the addition of glycine to the diet of patients with muscular dystrophy resulted in an extra creatine excretion, and those of Fisher & Wilhelmi (102), who found that the addition of arginine to the perfusion fluid of rabbit heart muscle yielded a quantitative amount of creatine. The latter authors (103, 104) suggested that glycine or glycolic acid serve as methylating agents.

As creatinine had been shown with the aid of N^{15} (105) to be directly and exclusively derived from creatine in the animal, both substances must have the same precursors. It was also reported that the conversion of creatine to creatinine is not reversible *in vivo*. Beard and associates (106, 107), however, still hold to the contention that creatine is not converted into creatinine, but that the reverse process occurs, i.e., formation of creatine from creatinine. This view is in disagreement with the results of all other investigators.

In agreement with the concept that guanidoacetic acid (glycocyamine) is an intermediate in creatine formation, the treatment of this compound with liver slices in the presence of methionine yields creatine (85). Furthermore, the feeding of isotopic guanidoacetic acid to normal animals leads to the rapid formation of isotopic creatine (108). The biological synthesis of this creatine, therefore, occurs in at least two steps: (a) the formation of guanidoacetic acid, and (b) methylation of the latter to creatine.

Reaction (b) (methylation) has already been reviewed in the section on transmethylation. The methyl groups of methionine and of choline have definitely been established as the source of the methyl group of creatine (84, 85, 86, 109). Borsook & Dubnoff (109) have shown that the site of methylation of guanidoacetic acid by methionine is restricted to the liver. Liver slices from all species so far investigated (cat, dog, guinea pig, frog, pigeon, rabbit, and rat) formed creatine while the kidneys, except those of pigeons, did not carry out the reaction. As the effect of methionine was small, considering the

[3] *Ann. Rev. Biochem.*, 4, 243 (1935).

concentration used, Borsook & Dubnoff suggest that the actual methylating agents for guanidoacetic acid may be a deamination product of methionine, rather than the amino acid itself.

The origin of the other part of the creatine molecule, i.e., guanido-acetic acid, has been investigated with the aid of heavy nitrogen, and with tissue slices. When isotopic tyrosine, leucine, glutamic acid, or ammonium citrate were given as a small addition to the stock diet of normal rats, the creatine isolated from the muscles, or the creatinine isolated from the urine, contained little or no marked nitrogen. These compounds thus cannot be regarded as direct creatine precursors. In contrast to the rather ineffective amino acids, glycine is a highly active creatine precursor (108). Administration of small amounts of its isotopic analogue gives rise to creatine with a relatively high isotope concentration. Sarcosine has almost the same effect as glycine. This substance is so rapidly demethylated that the glycine thereby liberated enters all the normal pathways of glycine metabolism (23), and it, therefore, is also used (indirectly) for creatine formation. In these investigations with heavy nitrogen, arginine is the only other amino acid which has been found to be a direct creatine precursor (110). The $l(+)$-arginine employed was synthesized in such a way that the isotope was present only in the amidine part (NH_2—C=NH),

i.e., in the same group which is recovered as urea when arginine is treated with arginase.

Glycine and arginine have different functions in creatine synthesis. The two isotopic creatine samples obtained after feeding isotopic glycine and isotopic arginine respectively, differed in isotopic constitution. Alkaline degradation and isotope analysis of the split products (ammonia and sarcosine) revealed that isotopic glycine feeding resulted in the formation of a creatine with isotope in the sarcosine part, while feeding of isotopic arginine led to a creatine with isotope in the amidine group. The sarcosine part of creatine (or the glycine part of guanido-acetic acid) was thus formed from glycine, and the latter was condensed with the amidine group transferred from arginine. The slight action of other amino acids as creatine producers is thus easily explained as an indirect effect. Their nitrogen was used for the formation of the amidine group of arginine, which in turn was transferred to creatine.

The interaction of glycine and arginine in creatine formation has also been studied independently by Borsook & Dubnoff (111) by the

method of tissue slices. When glycine and arginine were treated with surviving tissue slices of rat kidney, guanidoacetic acid was formed at a rate as rapid as that of the formation of urea from ammonia and ornithine in the liver. The identity of guanidoacetic acid was established by the resistance of its anhydride (glyocyamidine) to the specific enzyme of Dubos & Miller and by isolation and elementary analysis of its acetate. Liver slices, however, which were shown to carry out the methylation, are incapable of synthesizing guanidoacetic acid. It thus seems that the two stages of biological creatine synthesis are carried out in different organs: guanidoacetic acid, formed in the kidney, is methylated in the liver.

Another indication of the role of glycine as a precursor of creatine has been advanced by Almquist & Mecchi (19). Glycine, which is an indispensable amino acid in the diet of chicks, can be replaced by creatine (see page 203).

On the basis of the various experiments biological creatine synthesis is carried out according to the scheme:

$$
\begin{array}{cccc}
& & NH_2 & NH_2 \\
& & | & | \\
& & C{=}NH & C{=}NH \\
& & | & | \\
NH_2 & NH_2 & NH & NCH_3 \\
| & | & | & | \\
CH_2COOH + & -C{=}NH \rightarrow & CH_2COOH + & -CH_3 \rightarrow CH_2COOH \\
& \text{From arginine} & & \text{From methionine} \\
& & & \text{or choline}
\end{array}
$$
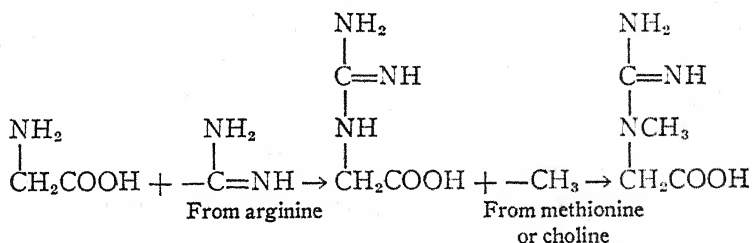
While it is, at present, not yet possible to state that the above scheme represents the only pathway of creatine formation, the occurrence of some other frequency discussed mechanisms can be excluded. Hydantoic acid, methyl hydantoic acid, and urea,[4] when given as their isotopic analogues, do not lead to the formation of isotopic creatine (108).

The new findings with tissue slices or isotopes cannot be reconciled with the theory of Beard and collaborators. According to Beard & Pizzolato (112) administration of almost all amino acids and many other compounds results in a large increase of muscle creatine and in

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[4] Urea is completely ineffective. None of the urea nitrogen of a sample with very high isotope content was recovered in the creatine after administration to rats (unpublished results).

extra creatine (or creatinine) excretion. They have put forward a theory according to which urea and glycine condense to guanido-acetic acid, which is methylated by another molecule of glycine. Fisher & Wilhelmi (104) have repeated the experiments with urea and glycine and were unable to confirm them. They suggest that the observed increase of muscle creatine is only apparent, as the massive doses of amino acids were not absorbed, and resulted in dehydration of the organs. They calculate from the values of Beard & Pizzolato on the action of prostigmine that 0.1 atom of hydrogen of the ad-ministered prostigmine has given rise to one atom in the newly found creatine.[5]

Beard, Espenan & Pizzolato (113) have also investigated the utilization of urea and glycine for creatine formation in experiments with humans. After the intake of 5 gm. of urea or 5 gm. of glycine "97 per cent of the theoretical amount of creatine and creatinine" was excreted. The intake of both compounds together resulted in an excretion of 94.6 per cent of the theory. The actual values given by the authors, however, indicate that each of the compounds has given rise simultaneously to about one mole of creatine and one mole of creatinine, i.e., to about 200 per cent of the theory. Even the authors' interpretation, however, cannot be reconciled with the fact (114) that administered urea is rapidly and almost completely excreted as such.

Beard & Espenan (115) have investigated creatine formation *in vitro* from urea with glycine or sarcosine in the presence of muscle tissue. The finding that the reaction proceeded even after the muscle had been autoclaved at 15 lb. was taken as indication that creatine formation does not require the presence of an enzyme. These authors have also reported that guanidoacetic acid is formed *in vitro* from glycine and urea in aqueous solution. However, the reaction product, which according to Lippich (116) is hydantoic acid, was not identi-fied by isolation.

DECARBOXYLATION

The optimum conditions for the decarboxylation of amino acids by microorganisms have been investigated in great detail by Gale.

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[5] Analogous calculations of the fate of the nitrogen of the administered com-pounds reveal that most amino acids tested had yielded more than 100 per cent of their nitrogen as creatine. This result would be comprehensible only if creatine, and not urea, were the chief end product of nitrogen metabolism.

Between pH 4 and 5, arginine, lysine, histidine, and glutamic acid are quantitatively converted by *B. coli* to agmatine, cadavarine, histamine, and α-aminobutyric acid respectively. There is evidence that each of the above reactions requires a specific decarboxylase (117). Strains of *Strep. faecalis* decarboxylate tyrosine to tyramine but do not produce amines from any other amino acids (118). This organism can, however, produce ornithine and ammonium carbonate from arginine. This reaction, which various streptococci can carry out, has been shown to be due to an enzyme other than arginase, since urea is not formed (119). The action of a mixture of *B. coli* and *Strep. faecalis* on arginine therefore produces ornithine, and on decarboxylation of these two amino acids agmatine and putrescine are formed. The combined effects of these two organisms thus explains conflicting reports in the earlier literature as to the products of bacterial action on arginine (120). The decarboxylation of the natural forms of histidine, tyrosine, and dioxyphenylalanine by extracts of organs from a variety of animals has been shown by Holtz, Credner & Walter (121) to be due to the action of different decarboxylases specific for each amino acid. The behavior and characteristics of histidine decarboxylase and of histaminase has been investigated by Werle (122).

The organism *Hemophilus parainfluenzae* has been shown by Klein (123) to oxidize $l(+)$-glutamic and $l(+)$-aspartic acids under suitable conditions. The reaction proceeds to acetic acid in both instances, with the production of carbon dioxide and ammonia. The fifteen other natural amino acids tested were found to be resistant.

MISCELLANEOUS PAPERS

Lack of space prevents more than brief mention of several papers dealing with various aspects of nitrogen metabolism. The well-known protein-sparing action of incomplete proteins (gelatin and zein) is explained by the assumption that tryptophane and other indispensable amino acids liberated by tissue catabolism are again utilized for the completion of inadequate proteins (124). Nitrogen equilibrium and even nitrogen gain in infants can be obtained by intravenous injection of pure amino acids (125) or by casein hydrolyzates (126, 127). Anaphylactic shock, produced experimentally in dogs, greatly increases nitrogen excretion, indicating tissue injury (128). When the protein and carbohydrate components of an otherwise adequate diet are fed separately at different times of the day to young rats, a loss of appetite results, but the ability to metabolize the proteins is not

impaired. In mature rats, and in humans, the procedure is accompanied by a small loss of nitrogen and body weight (129). An enzymatic digest of casein is nutritionally equal to casein itself both in growth promotion and in the regeneration of serum protein (130). A large variety of glutamine analogues and derivatives have been found incapable of replacing glutamine in supporting the growth of *Strep. haemolyticus* (131).

The effect of different levels of protein consumption by the adult rat has been studied with reference to physiological function (132); to energy and nitrogen metabolism (133); to protein content of organs and tissues (134); and to protein anabolism in the organs and tissues of pregnant rats (135). The effect of the consumption of various food proteins on protein deposition in the heart, kidney, and liver of previously starved rats was studied (136). The metabolism of tyrosine, aspartic acid, and asparagine has been investigated with special reference to respiratory exchange and heat production (137).

LITERATURE CITED

1. MADDEN, S. C., AND WHIPPLE, G. H., *Physiol. Rev.,* **20**, 194 (1940)
2. MADDEN, S. C., GEORGE, W. E., WARAICH, G. S., AND WHIPPLE, G. H., *J. Exptl. Med.,* **67**, 675 (1938)
3. MADDEN, S. C., FINCH, C. A., SWALBACH, W. G., AND WHIPPLE, G. H., *J. Exptl. Med.,* **71**, 283 (1940)
4. SCHOENHEIMER, R., AND RITTENBERG, D., *Physiol. Rev.,* **20**, 218 (1940)
5. RATNER, S., RITTENBERG, D., KESTON, A. S., AND SCHOENHEIMER, R., *J. Biol. Chem.,* **134**, 665 (1940)
6. MOSS, A. R., AND SCHOENHEIMER, R., *J. Biol. Chem.,* **135**, 415 (1940)
7. ROLOFF, M., RATNER, S., AND SCHOENHEIMER, R., *J. Biol. Chem.,* **136**, 561 (1940)
8. CLUTTON, R. F., SCHOENHEIMER, R., AND RITTENBERG, D., *J. Biol. Chem.,* **132**, 227 (1940)
9. VICKERY, H. B., PUCHER, G. W., SCHOENHEIMER, R., AND RITTENBERG, D., *J. Biol. Chem.,* **135**, 531 (1940)
10. HEVESY, G., LINDERSTRØM-LANG, K., KESTON, A. S., AND CARSTEN, O., *Compt. rend. trav. lab. Carlsberg. sér. chim.,* **23**, 213 (1940)
11. BURROUGHS, E. W., BURROUGHS, H. S., AND MITCHELL, H. H., *J. Nutrition,* **19**, 271 (1940)
12. SCHMIDT, C. L. A., ALLEN, F. W., AND TARVER, H., *Science,* **91**, 18 (1940)
13. DIRR, K., AND STREHLE, J., *Z. physiol. Chem.,* **260**, 56, 65 (1939)
14. DIRR, K., AND BADER, E., *Z. physiol. Chem.,* **264**, 135 (1940)
15. DIRR, K., AND LEIS, G., *Z. physiol. Chem.,* **263**, 216 (1940)
16. BLOCK, R. J., *J. Biol. Chem.,* **133**, 71 (1940)

17. Murrill, W. A., Block, W. D., and Newburgh, L. H., *J. Biol. Chem.,* 133, 521 (1940)
18. Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.,* 134, 213 (1940)
19. Almquist, H. J., and Mecchi, E., *J. Biol. Chem.,* 135, 355 (1940)
20. MacKay, E. M., Wick, A. N., and Carne, H. O., *J. Biol. Chem.,* 132, 613 (1940)
21. Wick, A. N., MacKay, E. M., Carne, H. O., and Mayfield, H. M., *J. Biol. Chem.,* 136, 237 (1940)
22. Abbott, Jr., L. D., and Lewis, H. B., *J. Biol. Chem.,* 131, 479 (1939)
23. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.,* 135, 99 (1940)
24. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.,* 132, 307 (1940)
25. Sealock, R. R., and Silberstein, H. E., *Science,* 90, 517 (1939)
26. Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.,* 135, 251 (1940)
27. Jervis, G. A., Block, R. J., Bolling, D., and Kanze, E., *J. Biol. Chem.,* 134, 105 (1940)
28. Block, R. J., Jervis, G. A., Bolling, D., and Webb, M., *J. Biol. Chem.,* 134, 567 (1940)
29. Evans, Jr., E. A., and Slotin, L., *J. Biol. Chem.,* 136, 301 (1940)
30. Felix, K., and Naka, S., *Z. physiol. Chem.,* 264, 123 (1940)
31. Krebs, H. A., *Z. physiol. Chem.,* 218, 157 (1933)
32. Cohen, P. P., *J. Biol. Chem.,* 136, 565 (1940)
33. Braunstein, A. E., *Enzymologia,* 7, 25 (1939)
34. Cohen, P. P., *J. Biol. Chem.,* 136, 585 (1940)
35. Harris, R. L., and Mattill, H. A., *J. Biol. Chem.,* 132, 477 (1940)
36. Sakaguchi, R., *J. Biochem. (Japan),* 31, 289 (1940)
37. Racker, E., *Biochem. J.,* 34, 89 (1940)
38. Neuweiler, W., and Grimm, W., *Klin. Wochschr.,* 19, 155 (1940)
39. Blamey, E. R., and Brand, E., *Proc. Soc. Exptl. Biol. Med.,* 42, 747 (1939)
40. Brand, E., Cahill, G. F., and Kassell, B., *J. Biol. Chem.,* 133, 431 (1940)
41. Sjoberg, K., and Nilsson, F., *Svensk Veterinärtid.,* 45, 197 (1940)
42. White, J., and White, A., *J. Biol. Chem.,* 131, 149 (1939)
43. Stekol, J. A., *Proc. Soc. Exptl. Biol. Med.,* 43, 108 (1940)
44. Bennett, M. A., *Biochem. J.,* 33, 1794 (1939)
45. Almquist, H. J., Mecchi, E., Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.,* 134, 465 (1940)
46. Arnold, A., Kline, O. L., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.,* 116, 699 (1936)
47. Klose, A. A., Stokstad, E. L. R., and Almquist, H. J., *J. Biol. Chem.,* 123, 691 (1938)
48. Klose, A. A., and Almquist, H. J., *J. Biol. Chem.,* 135, 153 (1940)
49. Burroughs, E. W., Burroughs, H. S., and Mitchell, H. H., *J. Nutrition,* 19, 363, 385 (1940)
50. Rose, W. C., *Physiol. Rev.,* 18, 109 (1938)
51. Whipple, G. H., and Robscheit-Robbins, F. S., *J. Exptl. Med.,* 71, 569 (1940)
52. Arnow, L. E., Opsahl, J. C., and Watson, C. J., *Proc. Soc. Exptl. Biol. Med.,* 43, 766 (1940)

53. Kögl, F., Erxleben, H., and Akkerman, A. M., *Z. physiol. Chem.*, **261**, 141 (1939)

54. Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E., *Nature*, **145**, 311 (1940)

55. Graff, S., Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 745 (1940)

56. Kögl, F., and Erxleben, H., *Z. physiol. Chem.*, **261**, 154 (1939)

57. Clark, W. G., Arnow, L. E., and Opsahl, J. C., *Proc. Soc. Exptl. Biol. Med.*, **43**, 767 (1940)

58. Schramm, G., and Müller, H., *Naturwissenschaften*, **28**, 223 (1940)

59. Kögl, F., Erxleben, H., *Z. physiol. Chem.*, **264**, 108 (1940)

60. Lipmann, F., Behrens, O. K., Kabat, E. A., and Burk, D., *Science*, **91**, 21 (1940)

61. Town, B. W., *Nature*, **145**, 312 (1940)

62. Konikova, A., *Nature*, **145**, 312 (1940)

63. Johnson, J. M., *J. Biol. Chem.*, **132**, 781 (1940)

64. Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E., *Biochem. J.*, **34**, 285 (1940)

65. Arnow, L. E., and Opsahl, J. C., *J. Biol. Chem.*, **133**, 765 (1940)

66. Johnson, J. M., *J. Biol. Chem.*, **134**, 459 (1940)

67. Behrens, O. K., Lipmann, F., Cohn, M., and Burk, D., *Science*, **92**, 32 (1940)

68. Arnow, L. E., and Opsahl, J. C., *Science*, **91**, 431 (1940)

69. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940)

70. Ratner, S., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **134**, 653 (1940)

71. Felix, K., and Zorn, K., *Z. physiol. Chem.*, **258**, 16 (1939)

72. Karrer, P., and Frank, H., *Helv. Chim Acta*, **23**, 948 (1940)

73. Axelrod, A. E., Sober, H. A., and Elvehjem, C. A., *Nature*, **144**, 670 (1939)

74. Ochoa, S., and Rossiter, R. J., *Nature*, **144**, 787 (1939)

75. Axelrod, A. E., Sober, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **134**, 749 (1940)

76. Rossiter, R. J., *J. Biol. Chem.*, **135**, 431 (1940)

77. Birkofer, L., and Wertzel, R., *Z. physiol. Chem.*, **264**, 31 (1940)

78. Bach, S. J., *Biochem. J.*, **33**, 1833 (1939)

79. Leuthardt, F., *Z. physiol. Chem.*, **252**, 238 (1938)

80. Munro, A. F., *Biochem. J.*, **33**, 1957 (1939)

81. Evans, Jr., E. A., and Slotin, L., *J. Biol. Chem.*, **136**, 805 (1940)

82. Rittenberg, D., and Waelsch, H., *J. Biol. Chem.*, **136**, 799 (1940)

83. Herrin, R. C., *Am. J. Physiol.*, **129**, 146 (1940)

84. Vigneaud, V. du, Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939)

85. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940)

86. Vigneaud, V. du, Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940)

86a. Vigneaud, V. du (Personal communication)

87. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **131**, 567 (1939)

88. GRIFFITH, W. H., AND WADE, N. J., *J. Biol. Chem.*, **132**, 627 (1940)
89. GRIFFITH, W. H., *J. Biol. Chem.*, **132**, 639 (1940)
90. GRIFFITH, W. H., *J. Nutrition*, **19**, 437 (1940)
91. GRIFFITH, W. H., AND MULFORD, D. J., *Proc. Soc. Exptl. Biol. Med.*, **45**, 657 (1940)
92. TUCKER, H. F., AND ECKSTEIN, H. C., *J. Biol. Chem.*, **121**, 479 (1937)
93. BEST, C. H., AND RIDOUT, J. H., *J. Physiol.*, **97**, 489 (1940)
94. CHANDLER, J. P., AND VIGNEAUD, V. DU, *J. Biol. Chem.*, **135**, 223 (1940)
95. CHANNON, H. J., MANIFOLD, M. C., AND PLATT, A. P., *Biochem. J.*, **34**, 866 (1940)
96. TUCKER, H. F., TREADWELL, C. R., AND ECKSTEIN, H. C., *J. Biol. Chem.*, **135**, 85 (1940)
97. CARTER, H. E., AND MELVILLE, D. B., *J. Biol. Chem.*, **133**, 109 (1940)
98. HOFMEISTER, F., *Arch. exptl. Path. Pharmakol.*, **33**, 198 (1893–94)
99. SCHULTZ, J., AND LEWIS, H. B., *J. Biol. Chem.*, **133**, 199 (1940)
100. HYDE, E., *J. Biol. Chem.*, **134**, 95 (1940)
101. BRAND, E., HARRIS, M. M., SANDBERG, M., AND RINGER, A. I., *Am. J. Physiol.*, **90**, 296 (1929)
102. FISHER, R. B., AND WILHELMI, A. E., *Biochem. J.*, **31**, 1136 (1937)
103. DAVENPORT, H. W., FISHER, R. B., AND WILHELMI, A. E., *Biochem. J.*, **32**, 262 (1938)
104. FISHER, R. B., AND WILHELMI, A. E., *J. Biol. Chem.*, **132**, 135 (1940)
105. BLOCH, K., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **131**, 111 (1939)
106. BEARD, H. H., AND JACOB, E. J., *Arch. Neurol. Psychiat.*, **42**, 67 (1939)
107. BEARD, H. H., AND JACOB, E. J., *Endocrinology*, **26**, 1064 (1940)
108. BLOCH, K., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **133**, 633 (1940)
109. BORSOOK, H., AND DUBNOFF, J. W., *J. Biol. Chem.*, **134**, 635 (1940)
110. BLOCH, K., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **134**, 785 (1940)
111. BORSOOK, H., AND DUBNOFF, J. W., *Science*, **91**, 551 (1940)
112. BEARD, H. H., AND PIZZOLATO, P., *J. Biochem. (Japan)*, **28**, 421 (1938)
113. BEARD, H. H., ESPENAN, J. K., AND PIZZOLATO, P., *Am. J. Physiol.*, **127**, 716 (1939)
114. KRISS, M., AND LAWSON, F. M., *J. Nutrition*, **19**, 151 (1940)
115. BEARD, H. H., AND ESPENAN, J. K., *Enzymologia*, **8**, 241 (1940)
116. LIPPICH, F., *Ber.*, **41**, 2976 (1908)
117. GALE, E. F., *Biochem. J.*, **34**, 392 (1940)
118. GALE, E. F., *Biochem. J.*, **34**, 846 (1940)
119. HILLS, G. M., *Biochem. J.*, **34**, 1057 (1940)
120. GALE, E. F., *Biochem. J.*, **34**, 853 (1940)
121. HOLTZ, P., CREDNER, K., AND WALTER, H., *Z. physiol. Chem.*, **262**, 111 (1939)
122. WERLE, E., *Biochem. Z.*, **304**, 201 (1940)
123. KLEIN, J. R., *J. Biol. Chem.*, **134**, 43 (1940)
124. LINTZEL, W., AND RICHENBERGER, J., *Biochem. Z.*, **304**, 214 (1940)
125. SHOHL, A. T., AND BLACKFAN, K. D., *J. Nutrition*, **20**, 305 (1940)
126. SHOHL, A. T., BUTLER, A. M., BLACKFAN, K. D., AND MACLACHLAN, E., *J. Pediat.*, **15**, 469 (1939)
127. ALTSHULER, S. S., HENSEL, H. M., AND SAHYUN, M., *Am. J. Med. Sci.*, **200**, 239 (1940)

128. MILLER, L. L., *J. Biol. Chem.,* **133,** 93 (1940)

129. CUTHBERTSON, D. P., McCUTCHEON, A., AND MUNRO, H. N., *Biochem. J.,* 34, 1002 (1940)

130. MUELLER, A. J., KEMMERER, K. S., COX, JR., W. M., AND BARNES, S. T., *J. Biol. Chem.,* **134,** 573 (1940)

131. McILWAIN, H., *Biochem. J.,* **33,** 1942 (1939)

132. SLONAKER, J. R., *Stanford Univ. Pub., Univ. Ser., Biol. Sci.,* **6,** No. 4 (1939)

133. FORBES, E. B., BLACK, A., THACKER, E. J., AND SWIFT, R. W., *J. Nutrition,* **20,** 47 (1940)

134. ADDIS, T., LEE, D. D., LEW, W., AND POO, L. J., *J. Nutrition,* **19,** 199 (1940)

135. POO, L. J., LEW, W., LEE, D. D., AND ADDIS, T., *J. Nutrition,* **19,** 505 (1940)

136. YUEN, D. W., POO, L. J., LEW, W., AND ADDIS, T., *Am. J. Physiol.,* **129,** 685 (1940)

137. KRISS, M., AND MARCY, L. F., *J. Nutrition,* **19,** 297 (1940)

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THE BIOCHEMISTRY OF THE NUCLEIC ACIDS, PURINES, AND PYRIMIDINES[1]

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The literature that has been surveyed in the present review is chiefly that of the past four years.[2] During this period, research on the characterization of nucleic acids has entered upon its eighth decade, yet the first problem to be posed, that concerning the nature of nucleic acids, still awaits a satisfactory solution.

With the exception of a few publications that escaped general notice, it has been believed universally that one type of nucleic acid, plant nucleic acid, was always to be found in the nuclei of plant cells, and that another type of nucleic acid, animal nucleic acid, was always contained in the nuclei of animal cells. Sufficient evidence has now appeared to show that this generalization is no longer tenable. Feulgen, Behrens & Mahdihassan (1), as well as Behrens (2), by the use of a flotation method whereby the nuclei of cells could be freed from cytoplasmic constituents, have been able to isolate animal nucleic acid from the nuclei, and plant nucleic acid from the cytoplasm of rye embryos. Delaporte & Roukhelman (3) have found that animal nucleic acid is present in the nucleus of yeast cells, and that the readily isolable plant nucleic acid of yeast is present in the metachromatic granules. Feulgen and co-workers believe that animal nucleic acid may be a constituent of all nuclei, regardless of origin, and that plant nucleic acid is a cytoplasmic rather than a nuclear constituent. In view of these and the earlier data, it is now clear that the nucleic acids must be classified first according to a known distinguishing characteristic of the molecules, and second according to origin. The most distinguishing characteristic components of the molecules appear to be the carbohydrates. That of the plant nucleic acids is d-ribose, while that of the animal nucleic acids is d-2-desoxyribose. Thus, thymonucleic acid, a

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[1] I wish to acknowledge my indebtedness to Dr. John J. Eiler, with whom I have discussed both the selection of papers and many of the points covered in this review.

[2] In order to restrict this review to the space that has been assigned, many important contributions pertaining to certain phases of the subject have been omitted.

so-called animal nucleic acid, should be termed desoxyribonucleic acid from thymus, and yeast nucleic acid, which is the classical example of a so-called plant nucleic acid, should be termed ribonucleic acid from yeast.

Chemistry of nucleic acids and their derivatives.—At the present time,[3] the solution to the problem of the structure of the nucleic acids is dependent upon more exact information concerning the following conditions: (*a*) the mode of union of the individual mononucleotides in the simple tetranucleotide molecule, (*b*) the relative position occupied by each mononucleotide in the nucleic acid molecule, and (*c*) the mode of union of the tetranucleotides in the formation of a polymerized molecule, providing, of course, that such a phenomenon is definitely found to occur in all types of nucleic acids.

In the molecule of ribonucleic acid it is generally considered that the mononucleotides are linked together to form the tetranucleotide structure by means of doubly linked phosphate radicals. The positions that are presumed to be occupied by the phosphate radicals are the second and third carbons of the ribose component. The allocation of similar linkages in the desoxyribonucleic acid molecule is at the third and fifth carbons of adjacent desoxyribose components. Gulland & Jackson (4) have noted that the degree of dephosphorylation of ribonucleic acid, under the action of various mixtures of phosphomonoesterases and phosphodiesterases, is 75 per cent, thus suggesting that one phosphate group may be constituted differently than the others. It was also noted that the joint action of phosphodiesterase and 5-nucleotidase liberates 35 per cent of the total phosphorus as inorganic phosphate; this suggests that two or more of the phosphate groups may be attached at position five of the ribose component. In a later work by the same authors (5), it is claimed that evidence is obtained whereby the phosphatase-resistant group may be said definitely to be associated with the cytidylic and adenylic acid radicals. This possibly may indicate that the phosphate radical is substituted for a hydrogen of an amino group or of the hydroxyl of the fifth carbon of the ribose. Support for the existence of a phosphorus-nitrogen linkage between the amino group of guanine and the phosphoric acid group of uridylic acid was offered by Bredereck & Richter (6), who, by boiling an aqueous solution of ribonucleic acid, isolated a substance believed to be guanine-uridylic acid. The suggestion of the presence of a phosphorus-nitrogen

[3] Recent reviews which give extensive surveys of the earlier periods of research have been written by Gulland and by Bredereck (3a).

union in ribonucleic acid was later withdrawn when Bredereck, Köthnig & Lehmann (7) subjected ribonucleic acid to deamination. During the course of the deamination, no cleavage to mononucleotides was observed, and it was shown that the product still retained a polynucleotide structure. Hence, guanine-uridylic acid was considered not as a structural unit of ribonucleic acid, but as a secondary product arising during the hydrolysis. Tipson & Levene (8) repeated the earlier work of Bredereck & Richter and were unable to obtain sufficient evidence to support the view. Falconer, Gulland, Hobday & Jackson (9, 10) refuse to accept the conclusions of Bredereck, Köthnig & Lehmann, that guanine-uridylic acid is necessarily a secondary product, or the conclusions of Tipson & Levene, that sufficient evidence has not yet been furnished in support of the view that the individual nucleotides may be linked through phosphorus-nitrogen linkages in ribonucleic acid. In an exhaustive study the above authors were unable, in six attempts, to isolate guanine-uridylic acid from the hydrolytic products of the ribonucleic acid furnished by a British firm. Positive results were obtained, however, whenever ribonucleic acid furnished by either Merck or Boehringer (German firms) was hydrolyzed. This implies the existence of two types of ribonucleic acid which may be formed as a result of different isolation procedures. It is also shown that guanine-uridylic acid is not stable when subjected to deamination for thirty minutes at 20°; hence, the linkage could not be expected to remain intact when ribonucleic acid is subjected to deamination. It is emphasized that the linkage between guanine and uridylic acid is not the heretofore mentioned group (5) that is resistant to enzymic cleavage.

Conclusions drawn concerning the structure of desoxyribonucleic acid are often introduced when considering the structure of ribonucleic acid and vice versa. Hence, the results of studies on the enzymic cleavage of the two type nucleic acids should be cited here. Bredereck, Caro & Richter (11) have studied the rate of hydrolytic cleavage of desoxyribo- and ribonucleic acids into nucleotides and nucleosides by the use of an enzyme-containing extract from sweet almonds. The enzyme is active at pH 4.5 to 5.1. Inorganic phosphate was found to be liberated from both nucleic acids at approximately the same rate. The conclusion is drawn that the nature of the unions between the nucleotides in both nucleic acids is the same. In an extension of the investigation to include the enzymic action that takes place in alkaline medium, Bredereck & Müller (12) used an enzymic preparation from the intestinal mucosa of the calf. In this instance the cleavage of ribo-

nucleic acid was noted to occur more rapidly than that of desoxyribonucleic acid. This is in agreement with earlier work by Levene & Dillon, but not in accord with that of Klein.[4] As will be pointed out later, it may well be that the existence of conflicting data concerning the enzymic cleavage of nucleic acids at alkaline reactions may be attributed to the degree of polymerization of the nucleic acid that is employed.

In answer to the difficult question regarding the relative positions of the nucleotides in a tetranucleotide structure, no further progress has been made. The pyrimidine nucleotides are stable under conditions that cause the hydrolysis of the purine nucleotides; hence, one of the principal arguments in favor of allocating the purine and pyrimidine nucleotides in alternating positions, as is generally accepted, is based upon the reported isolation of diphosphoric esters of pyrimidine desoxyribosides from desoxyribonucleic acid by Levene & Jacobs (13) and by Thannhauser & Ottenstein (14). These findings have been placed under attack by Bredereck & Caro (15) who claim that indisputable evidence for the existence of such esters does not exist. Levene (16) has successfully met these claims by reanalyses of the old material upon which the evidence was based and has shown that the analytical results still support the original conclusions concerning the existence of the diphosphoric acid esters. While not interpreted as such by the authors, further positive evidence, of an indirect nature, in support of the existence of the diphosphoric esters may be claimed from the work of Bredereck & Müller (17), who have announced the isolation of thymic acid from desoxyribonucleic acid, thus confirming the claims of other earlier workers.

Exact information regarding the existence of the nucleic acids in polymerized forms is of rather recent introduction. There is now ample evidence to show that desoxyribonucleic acid may exist in a highly polymerized state. Signer, Caspersson & Hammarsten (18) have concluded, from studies of the viscosity and double refraction of flow of desoxyribonucleic acid, that the molecular weight lies between 500,000 and 1,000,000. These figures have been confirmed by Astbury & Bell (19) in their study of the x-ray fiber photographs on a sample of desoxyribonucleic acid furnished by the former investigators. In place of the four mononucleotides usually considered to be the constituents of the molecule, the foregoing data indicate that at

[4] *Ann. Rev. Biochem.*, 2, 117 (1933); 4, 175 (1935).

least 2,000 mononucleotides enter into the structure of the sample that was under investigation. That all samples of desoxyribonucleic acid do not show the same degree of polymerization has been pointed out recently by Schmidt and co-workers (20, 21). According to studies on enzymic dephosphorylation, the degree of polymerization depends, to a large extent, upon the methods of preparation. The following procedures yield desoxyribonucleic acid whose degree of polymerization is of descending order: (a) Hammarsten (22), (b) Newmann (23), as modified by Feulgen (24), (c) Levene (25), and (d) Feulgen (26).

The extent to which ribonucleic acid may occur in a polymerized state is not clear. In fact, the experimental data heretofore published do not indicate a high degree of polymerization, if any. However, recent activity that centers around the chemical changes occasioned in the molecule of ribonucleic acid by a thermostable enzyme of the pancreas, first recorded by Jones (27) in 1920, has directed attention toward the possibility of polymerization in the ribonucleic acid molecule. The enzyme and its action have received recent attention at the hands of Dubos (28), Dubos & Thompson (29), Schmidt & Levene (30), Kunitz (31), and Allen & Eiler (32). Kunitz has isolated the enzyme in the crystalline state.

The chemical changes induced by the enzyme, as studied by Dubos & Thompson and by Schmidt & Levene, are such that the latter authors have suggested the function of the enzyme to be that of a depolymerase, "limited to the dissociation of the tetranucleotides of high molecular weight into those of lower molecular weight." The production of mononucleotides in the reaction was ruled out by the absence of products that would diffuse through membranes, as well as the absence of changes in the freezing point lowering of solutions subjected to the action of the enzyme. In contradiction of Schmidt & Levene, Kunitz has found that ribonucleic acid is split into fragments that are small enough to diffuse readily through collodion or cellophane membranes. Allen & Eiler have studied the increase in equivalents of acid that is effected by the enzymic action. In contradiction of Levene & Simms (33), and in agreement with Makino (34), and Gulland and co-workers (35), the number of equivalents of acid displayed by native ribonucleic acid within the range of the dissociable phosphoric acid groups has been found to be four per mole. The action of the enzyme effects the liberation of one equivalent of acid per mole. Data place the liberated acidic group in the range of a secondary phosphoric acid dissociation. When these data are examined to ascertain their bearing

on the structure of ribonucleic acid, it is found that they are compatible with either (*a*) the open chain structure proposed by Levene & Simms (33), provided a highly polymerized molecule is assumed wherein the secondary hydroxyl of a phosphate group is involved in the polymerization, or (*b*) an opening of the cyclic structure proposed by Takahashi (36).

A recent examination of the acid equivalency of desoxyribonucleic acid by Bredereck & Köthnig (37) confirms the earlier work of Levene & Simms (33) that the acid is pentabasic. However, the question as to whether the native acid, as opposed to the isolated acid, may not possibly be of a lower acid equivalency is yet to be settled. In this regard it should be emphasized that the known methods for the isolation of desoxyribonucleic acid subject the material to very drastic treatment with alkali, a treatment that may well bring about the liberation of a hitherto linked secondary phosphoric acid group.

Other phases of the chemistry of the nucleic acids and their derivatives are represented in the following reports. Tipson (38) has investigated the constitution of the acridine salts of the adenylic acids, isolated from muscle and from ribonucleic acid. They are shown to have the formula, $C_{13}H_9N \cdot 2C_{10}H_{14}O_7N_5P$, which is not in agreement with the formula, $C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P$, as recorded by Wagner-Juaregg (39). The presence of a new pyrimidine nucleotide in the ribonucleic acid from tobacco mosaic virus seemingly provides evidence for a new type of nucleic acid. Loring (40) believes that the uridylic acid, isolated as the brucine salt from a sample of ribonucleic acid from tobacco mosaic virus, is isomeric rather than identical with the uridylic acid isolated from the ribonucleic acid from yeast. Earlier studies by Gulland and co-workers[5] on the ultraviolet absorption spectra of the purine ribosides have established that position 9 of the purine, and not 7, as originally assumed by other workers, is the place of substitution of the ribose. This assumption is based on comparative studies of the ultraviolet absorption spectra of 7-methyl and 9-methyl substituted purines. The methyl group and the carbohydrate group are assumed to occupy the same positions when the spectra are closely similar and mutually unlike other methylated derivatives. The validity of the assumption is based upon the recognized fact that the effect of the carbohydrate group on the spectra is negligible. To the list of derivatives known to be substituted in position 9, Gulland and co-workers

have now added adenine thiomethyl pentoside (41), adenine desoxy-riboside (42), and guanine desoxyriboside (43). Stevens (44) has submitted evidence to show that the formic acid that is formed during the hydrolysis of nucleic acids by mineral acids has it origin in the decomposition of adenine and, to a minor extent, in guanine. It is believed by Semmens (45) that the Feulgen nucleal reaction can no longer be considered to be due solely to the potential aldehyde group of the aldose component of nucleic acids, but that adenine and guanine, as well as certain other purines, give the test. This is refuted by Barber & Price (46) who fail to confirm Semmens' results.

Certain physicochemical aspects, other than those mentioned in the foregoing pages, have received treatment at the hands of various investigators. Stenhagen & Teorell (47) have studied the electrophoretic properties of desoxyribonucleic acid from thymus. The x-ray spectra have been studied by Mazza & Tappi (48). The ultraviolet absorption spectra of the ribonucleic acid from tobacco mosaic virus have been studied by Lavin, Loring & Stanley (49). Koyenuma (50) has studied the solubility of desoxyribonucleic acid in water. The viscosity and streaming birefringence of desoxyribonucleic acid have been studied by Greenstein & Jenrette (51).

An outstanding characteristic that may be noted among many of the investigations concerning nucleic acids is the rather frequent lack of agreement between the results from different laboratories. The nucleic acids are known only as amorphous substances of questionable homogeneity. The molecules are composed of many components whose covalent linkages exhibit varying degrees of stability. In addition to the types of linkages already known to exist, the period under review has provided evidence that seemingly indicates the presence of other linkages. For this reason it is felt that the contrary findings are more apparent than real. The solution to the problem resides in the criteria that are imposed in order to establish the homogeneity of a given preparation. The sole criterion that may be used at the present time is that of the nitrogen and phosphorus content. The inadequacy of such a single criterion is obvious and does not warrant further discussion.

Preparation of purines, pyrimidines, nucleosides, and nucleotides.— With few exceptions, the isolation of the products of the hydrolysis of nucleic acids has necessitated the use of laborious, expensive procedures that gave very low yields. During the period under review many excellent methods have appeared that have made the heretofore difficulty available substances easily available in quantity. Hunter &

Hlynka (52) have shortened and modified the older well-known methods of Jones and of Levene in a manner such that guanine, adenine, cytosine, and uracil can all be prepared from the same sample of nucleic acid. The yields of cytosine and uracil are not quite so large as could be desired. Bredereck & Rothe (53) have used a preparation of phosphatase from sweet almonds in order to effect the hydrolytic cleavage of ribonucleic acid into its component nucleosides. If time is a limiting factor, the nucleic acid is first subjected to mild alkaline hydrolysis, thus liberating nucleotides. After proper adjustment of the pH to approximately pH 5.0, the subsequent hydrolysis to the nucleosides is relegated to the phosphatase. Guanosine may be isolated practically quantitatively, while adenosine is easily isolated in the usual manner as the picrate. If it is desired to isolate the cytidine or uridine from the digest, the older method of Levene & La Forge (54) must still be used. The method of Bredereck & Rothe has given excellent yields in the laboratory of the reviewer.

Adenosine, while relatively easy to obtain in the form of its picrate, has always been difficultly available because of the large losses that are encountered in the removal of the picric acid. The usual methods for the removal of picric acid have employed organic solvents, methods that at best are very time-consuming. Bredereck (55) removes the picric acid as the potassium salt, thereby greatly decreasing the loss of adenosine. A method, similar to that of Bredereck & Rothe, in which a phosphatase preparation from new potatoes is used to effect the hydrolysis, has been developed by Hartmann & Bosshard (56). Klein (57) reports that he has been able to increase the yields of desoxyribosides obtained from desoxyribonucleic acid by the substitution of ammonium buffers for the acetate buffers that were employed in a previously described method.

In a series of papers, Ostern and co-workers (58) describe methods whereby the enzymic synthesis of adenosine-5-phosphoric acid, as well as adenosine triphosphoric acid, may be effected by the use of fresh brewers' yeast or of yeast dried with acetone. The substrate for the preparation is adenosine. The yield for adenosine-5-phosphoric acid, based on the amount of adenosine that is added, is approximately 35 per cent. While fresh yeast does not contain adenosine, it is claimed that 60 per cent of the adenosine that is a component part of the nucleic acid normally present in yeast becomes free during twenty hours of autolysis; hence, the synthesis also may be carried out by the use of autolyzed yeast as the substrate. A new method for the isolation of

the pyrimidine nucleotides, cytidylic and uridylic acids, that avoids the time consuming recrystallization of the mixed brucine salts hitherto employed, has been published by Bredereck & Richter (59). Nucleic acid is boiled with a 2 per cent solution of sulfuric acid. This treatment hydrolyzes the purine nucleotides into simple components, but leaves the more stable pyrimidine nucleotides intact. After the removal of purines and the sulfate and phosphate ions, the pyrimidine nucleotides are separated by their differential solubility in pyridine. Cytidylic acid is precipitated from a cold concentrated aqueous solution by pyridine, while uridylic acid remains in solution. It is felt by the reviewer that a procedure such as that just described has excellent possibilities for filling a much needed requirement. However, in this laboratory, very low yields of the pyrimidine nucleotides were obtained by the use of the method.

As recorded in this paragraph, the partial synthesis of several of the nucleotides has been reported. However, in all cases, the yields are exceedingly low. Jachimowicz (60) describes the synthesis of adenosine-5-phosphoric acid from adenosine and phosphorus oxychloride. Levene & Tipson (61) have carried out the same synthesis by the phosphorylation of diacetyl adenosine, with the subsequent hydrolysis of the product to give adenosine-5-phosphoric acid. Bredereck, Berger & Ehrenberg (62) claim that direct phosphorylation of adenosine by the use of phosphorus oxychloride gives rise to a mixture of phosphoric acid esters. They have confirmed the claims of Levene & Tipson regarding the phosphorylation of diacetyl adenosine. Further, if diphenyl phosphorus oxychloride is used as the phosphorylating agent, and diacetyl adenosine as the substrate, the intermediary product, after hydrolysis, invariably yields adenosine-5-phosphoric acid. Cytidylic acid may be prepared and isolated as the brucine salt by employing trityl cytidine in the same type of reaction. In a like manner, Gulland & Hobday (63) report the synthesis of uridylic acid, guanylic acid, uridine-5-phosphoric acid, and guanosine-5-phosphoric acid.

Recently characterized purine or pyrimidine derivatives.—The isolation and characterization of many substances, whose existence had heretofore been known or postulated from physiological behavior, have added several more purine- or pyrimidine-containing substances to the number already known. Codehydrogenase I, under various other names, has received widespread attention for several decades. It is now known to be an adenine-containing nucleotide and has been

termed diphosphopyridine nucleotide. On the basis of chemical evidence, Euler & Schlenk (64) have proposed the following formula:
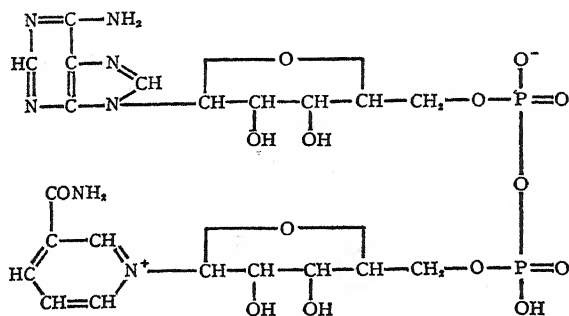
Diphosphopyridine nucleotide is readily soluble in water and stable at 80°. The action of alkali effects hydrolysis at the following three linkages: (a) the nicotinic acid amide-pentose linkage, (b) the nicotinic acid amide nucleotide-phosphorus linkage, and (c) the pyrophosphate linkage. A $0.1\ N$ solution of sodium hydroxide will attack linkage (a) at room temperatures, while linkages (b) and (c) require the use of $0.2\ N$ sodium hydroxide for five minutes or longer at 100° to effect hydrolysis (65, 66, 67). Adenosine-5-phosphoric acid may be obtained by the use of mild acid hydrolysis but, owing to the formation of adenine and nicotinic acid amide in the reaction, the yield is low (68). Enzymic decomposition by dephosphorylation is catalyzed by the same enzyme or enzymes that dephosphorylate adenylic and inosinic acids (69). The nicotinic acid amide nucleoside component of the molecule has been isolated and certain of its properties have been described by Schlenk (70). The component of the molecule that is most reactive physiologically is the nicotinic acid amide, which hydrogenates very rapidly in a reversible reaction.[6] Among the recent procedures for the isolation of diphosphopyridine nucleotide are those of Meyerhof & Ohlmeyer (71), Ochoa (72), Klein (73), and Williamson & Green (74).

Codehydrogenase II, earlier known as Warburg's coferment, has been characterized recently and is now termed triphosphopyridine nu-

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[6] The physiological actions of certain of the purine- and pyrimidine-containing derivatives have been reviewed in two papers (70a).

cleotide. The purine component is known to be adenine. The chemical behavior of triphosphopyridine nucleotide is almost identical with that of diphosphopyridine nucleotide. It is believed that the structure may be represented by the following expression (64, 75):
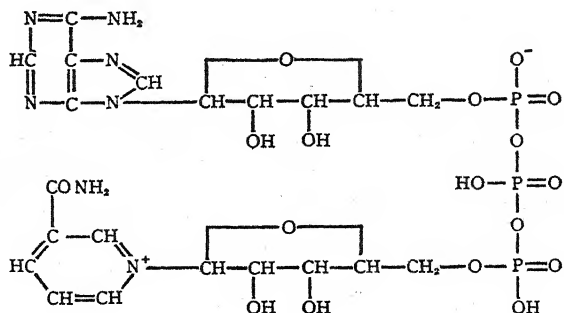
Triphosphopyridine nucleotide may be obtained from diphosphopyridine nucleotide by enzymic transformation, as shown by Euler and co-workers (76, 77).

Cocarboxylase has been isolated in crystalline form from brewers' yeast and is found to be composed of one molecule of thiamin and two molecules of phosphoric acid combined in the form of pyrophosphate (78). The pyrimidine component is known to be 2,5-dimethyl-6-amino pyrimidine. The formula of thiamin-pyrophosphate may be represented by the following structure:
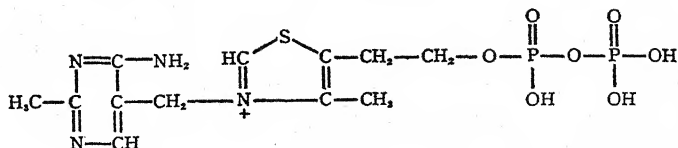
One molecule of phosphate is very labile toward acid hydrolysis, the other is more resistant. One of the most useful chemical reactions that is displayed is the oxidation of thiamin by potassium ferricyanide in alkaline solution to thiochrome. This reaction is the basis for several chemical procedures for quantitative estimation. The modifications and use of these procedures are described and discussed by Hennessy & Cerecedo (79).

The enzymic synthesis of thiamin-pyrophosphate by the use of bottom yeast and thiamin, in which the phosphate donor was adenosine triphosphate, has been reported by Euler & Vestin (80). Other enzymic syntheses from thiamin are those of Tauber (81) by the use of dried yeast and orthophosphate, or the duodenal mucosa of the pig, and of Ochoa (82) by the use of pigeon liver. Chemical syntheses by the interaction of phosphorylating reagents upon thiamin are claimed by Stern & Hofer (83) and by Tauber (84). No one of the foregoing methods has proved practical for isolation in quantity.

The coenzyme of the d-amino acid oxidase, first demonstrated in a biological system by Krebs (85), has been isolated by Warburg & Christian (86) and found to be alloxazine-adenine-dinucleotide. The alloxazine component is present in the molecule as its tautomeric form, isoalloxazine. The following structure may be assigned to it.
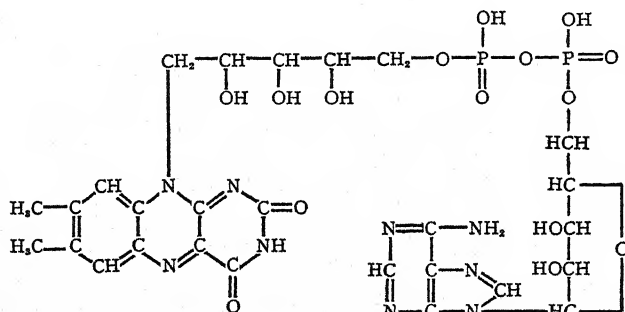
Alloxazine-adenine-dinucleotide, when joined to various specific proteins, forms (a) the d-amino acid oxidase (85), (b) the xanthine oxidase (87), and (c) pyridine nucleotide oxidases (88, 89, 90).

Procedures for the isolation of alloxazine-adenine-dinucleotide from liver, kidney, and yeast are given by Warburg & Christian (86), and from milk by Ball (87). The synthesis of alloxazine-adenine-dinucleotide from riboflavin by human red blood corpuscles in experiments in vitro and in vivo has been demonstrated by Klein & Kohn (91).

Fresh yeast has been found to contain adenosine-5-phosphoric acid in a combined form claimed to be di(adenosine-5-phosphoric acid). Kiessling & Meyerhof (92) have isolated a substance from the trichloroacetic acid filtrate of fresh beer yeast, which, when subjected to phosphorylation by the use of dialyzed muscle extracts containing

phosphopyruvic acid, yields di(adenosine-5-phosphoric acid)-pyro-phosphate. The substance behaves similarly to adenosine triphosphate as a phosphate donor. Two of the phosphate groups that are contained in the molecule are labile toward hydrolysis, while two are found to be resistant. Electrometric titration data show four acidic groups in the range of the primary phosphoric acid dissociation, and one in the range of the secondary phosphoric acid dissociation. On the basis of these and other chemical evidence, the following structure is assigned:
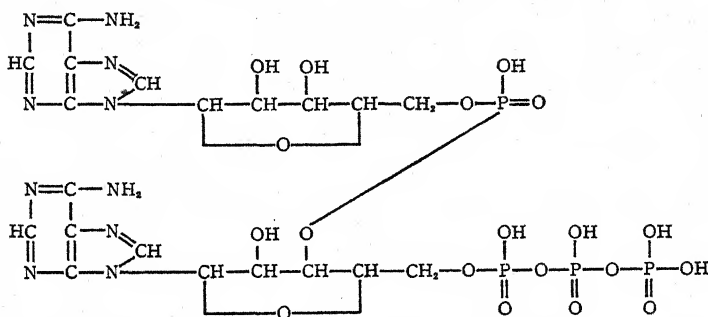
Uric acid riboside, first isolated from beef blood by Davis, New-ton & Benedict (93), has been found to be among the substances that are present when liver is permitted to autolyze at pH 5.0. A method for the isolation of the nucleoside and a description of certain of its properties are given by Falconer & Gulland (94). Evidence for the position of the glycosidic linkage is given and, if by analogy with the other purine nucleosides a furanose structure is assumed for the ribose component, the following structure may be assigned:
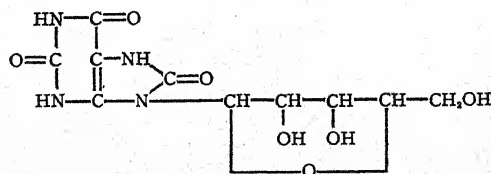
The structures for the recently isolated adenine-containing sub-

stances have been based partly on the claims of Lohmann[7] for the establishment of the structures of the adenosine polyphosphoric acids. The acceptance of these claims is widespread, but by no means universal. Barrenscheen & Jachimowicz (95) have re-examined certain of the earlier investigations of Barrenscheen and co-workers. It is believed that the rate of deamination of adenosine triphosphate, as well as the behavior toward bone phosphatase under definite experimental conditions, is not compatible with the structure advanced by Lohmann. Should these and earlier contentions prove correct, a re-examination of the structures of all of the substances containing adenosine polyphosphoric acids would then be in order.

Analytical methods.—The unsatisfactory state of the analytical methods in this field is shown by the number of papers that appear bearing proposals for modifications in the existing methods. Many of the modifications that are offered are concerned solely with matters of personal preference with regard to techniques, hence, no attempt is made to mention all of the papers that have come to the attention of the reviewer.

The question of the development of turbidity in the determination of uric acid when applying the Benedict methods for use with a photoelectric colorimeter, is considered and discussed by Diller (96). Directions for the preparation of a new chromogenic arsenotungstate and its use in the determination of the uric acid of blood are given by Newton (97). The color of the reduced reagent is said to be a pure blue. Also, it is emphasized that the reagent can be used only for determination in suitably fractionated biological fluids. A submicromethod for the determination of uric acid with the aid of a Pulfrich photometer is described by Florkin (98). Brøchner-Mortensen (99) has proposed a uric acid method that is essentially a modification of the Hagedorn & Jensen procedure. The results from large numbers of analyses of the blood and urine for many varying conditions are also given. A further discussion and comparison of uric acid procedures and analytical results are given by Brøchner-Mortensen in a recent extensive review (100). An interesting proposal of Blauch & Koch (101) is concerned with the use of uricase in conjunction with colorimetric methods for the determination of uric acid. The chromogenic value of a filtrate from blood that has had the uric acid destroyed by uricase is subtracted from the chromogenic value that is obtained for untreated blood, in order to obtain the true value for uric acid.

[7] *Ann. Rev. Biochem.,* **2,** 121 (1933); **4,** 178 (1935).

Approximately two-thirds of the color that is obtained by the direct reduction of phosphotungstate is due to uric acid. The remainder of the color originates from the oxidation of other substances that are not destroyed by uricase, such as glutathione, phenols, ascorbic acid, glucose, tyrosine, tryptophane, cystine, and cysteine. This method, when properly employed, should result in the clarification of many ideas concerning the uric acid of the blood, in much the same manner as has the employment of fermentation methods in the estimation of the glucose content of the blood.

The Fosse colorimetric method for the estimation of allantoin is based upon the conversion of allantoin into allantoic acid and the subsequent hydrolysis of the allantoic acid to form glyoxylic acid. Glyoxylic acid, when treated with a reagent containing phenylhydrazine, hydrochloric acid, and potassium ferricyanide, yields an intensely red-colored solution. This method and certain of its modified forms have been studied by Bosson (102) with a view toward adapting it to photometric measurement. Paget & Berger (103) have discussed the method and its use for the determination of allantoin in human urine. Florkin & Bosson (104) use the reaction in order to determine submicro amounts of allantoic acid. Schaffer & Greenbaum (105) describe a method whereby allantoin is precipitated from urine as the mercury salt, and subsequently determined as nitrogen by a micro-Kjeldahl procedure.

Parnas & Umschweif (106) have determined the furfural that is liberated from the ribose component of the adenosine phosphoric acids. The amount of furfural is found to be never more than 40 per cent of the calculated, and is dependent upon the concentration of sulfuric acid that is used to effect the conversion. Earlier methods that are based upon the furfural procedure are criticized and an improved technique is offered. Peham (107) has examined the method developed by Kerr & Blish (108) for the separate determination of the nucleotide and nucleoside plus free purine nitrogen in blood and other tissues, and has suggested that slight modifications be incorporated in the procedure. Also, Kerr & Seraidarian (109) have submitted the method of Kerr & Blish to an extensive examination and have been able to simplify and shorten many of the steps that are involved. Further, the original procedure has been extended in order to permit the separate estimation of adenine and hypoxanthine. The methods of Kerr and co-workers have been used in the laboratory of the reviewer (110, 111) and have been found to be most satisfactory. All results are

readily reproducible. However, recent observations regarding the mode of action of ribonuclease, together with the possible ubiquitous presence of ribonucleic acid in cytoplasm, require that caution in the interpretation of results is imperative. The action of ribonuclease on ribonucleic acid forms a polynucleotide that is soluble in trichloroacetic acid, and is precipitated with uranium in the same manner as the simple nucleotides (111a).

The Bial reaction for pentoses has been applied by Mejbaum (112) for use as a photometric method for the determination of micro amounts of the adenosine phosphoric acids. A modification for the determination of adenosine triphosphate by the method of Parnas & Lutwak-Mann (113) by the use of the barium salt is given by Conway & Cooke (114). Among a series of methods designed by Borsook & Dubnoff (115) for determining the partition of nitrogen between various nitrogen-containing fractions, adenylic acid is estimated separately. A method that uses the Kruger & Schmidt principle for separating purines from other nitrogen-containing substances has been described by Raekallio (116) for the microdetermination of the purine nitrogen of the urine. According to Krebs & Örström (117), hypoxanthine and xanthine may be estimated manometrically. The xanthine and hypoxanthine are converted into uric acid by the use of xanthine oxidase.

Metabolism.—The formation of ammonia that is noted to occur in shed blood has been ascribed to various purine-containing substances. Mozolowski (118) believes that a part of the ammonia is due to the deamination of nucleotides. The experiments of Mozolowski were of rather long duration in which hemolysis and its accompanying changes may have been an important factor. Heller & Klisiecki (119) have shown that, at least for sheep blood, deamination occurs only after hemolysis of the red blood corpuscles has taken place. Recently, Conway & Cooke (120) find that the ammonia of shed blood is formed in successive stages and is due mainly to the presence of adenosine deaminase. The stage of ammonia formation most pertinent to the present discussion is the "beta" stage of from three to five hours' duration, which follows the "alpha" or immediate stage. The ammonia that arises during the "beta" stage is mainly from adenosine. Of the fifty-one substances of physiological importance that were studied, only the following yielded ammonia in mammalian blood: adenosine, adenylic acid, adenosine triphosphate, and desoxyribonucleic acid (small amounts). Drury, Lutwak-Mann & Solandt (121) likewise have re-

ported that an enzymic system, present in the blood of the cat, inactivates adenosine with the liberation of ammonia. Muscle adenylic acid and adenosine triphosphate are also inactivated by blood plasma, but less readily than adenosine. Claiming support for Mozolowski rather than Heller & Klisiecki, Kerr & Antaki (122) find that the adenosine triphosphate present in trichloroacetic acid filtrates from blood or in laked blood loses two phosphate groups by phosphatase action, and is deaminized within ninety minutes. In studies concerning the relationship that may exist between glycolysis and the catabolism of purine nucleotides in the blood of the rabbit, Eiler & Allen (111) were unable to note any large percentage loss in total purine nitrogen during the progress of active glycolysis. It was therefore concluded that the rate of catabolism of the purine nucleotides in blood depends on the rate of glycolysis. During glycolysis it is expected that small amounts of adenylic acid must escape resynthesis to adenosine triphosphate, and thus fall into the catabolic phase which involves dephosphorylation and deamination.

Blauch & Koch (123) have used the previously described (101) specific uricase method for the determination of uric acid in an investigation of the blood of man, dog, rabbit, rat, guinea pig, and mouse. The following conditions were studied: (a) the uric acid content immediately after drawing, (b) the changes that occur in the uric acid content on incubation, and (c) the presence of agents causing the disappearance or the formation of uric acid. Although the destruction of uric acid has been reported at various times, Blauch & Koch were unable to confirm such reports. The uric acid content of human, dog, and rabbit blood, when oxalated and stored in the refrigerator or when incubated for several hours, alone or with the addition of adenine, guanine, xanthine, or hypoxanthine, was found to be constant. The uric acid content of rat and guinea pig blood increased rapidly on standing. No evidence was found for the presence of a uric acid complex from which uric acid might be formed. The incubation of rat and guinea pig blood with guanine, xanthine, or low concentrations of hypoxanthine produced increased uric acid contents. Hence, rat and guinea pig blood are concluded to contain guanase and xanthine oxidase. The addition of adenine or of hypoxanthine in high concentrations to rat blood inhibits the formation of uric acid (124), but adenine in low concentrations, if adequate time is permitted, will yield increased uric acid values. This indicates the presence of adenase in the blood of the rat.

Concerning the ultimate fate of the nucleic acids that are present in the leucocytes and reticulocytes, the following communications are of interest. The lymphocytes, according to Reding (125), are found to contain enzymes that effect the liberation of phosphate and purines from ribonucleic and desoxyribonucleic acids. Opsahl (126) has noted an increased excretion of uric acid during the reticulocyte crisis in five cases of pernicious anemia and in two cases of hemorrhagic anemia. A relationship between endogenous uric acid and the regeneration of the formed elements of the blood is postulated.

The role of the liver and kidney in the metabolism of purines has been emphasized mainly through studies concerning the enzymic systems of uricase and xanthine oxidase. Allantoicase, heretofore known to be present in the plant kingdom, has been shown by Brunel (127) to occur in the livers of several species of the genera *Raja* and *Rana*.

Uricase has been prepared from pig liver by Davidson (128) in a form that is 550 times more active than earlier preparations. Extremely low values are obtained for the iron content of the purest preparations (128, 129), and no evidence can be found to support the assumption that the iron present may act in the capacity of an active group. An exceedingly interesting observation concerning the action of uricase has been reported by Ball (87). It is found that the end product of hypoxanthine oxidation by purified xanthine oxidase does not act as a substrate for uricase. However, when crude preparations of xanthine oxidase are used, an end product that is oxidized by uricase is produced. An explanation for this difference cannot yet be offered.

With the exception of the anthropoid ape, the Dalmatian coach hound has been considered to be the only mammal similar to man regarding the end products of purine metabolism. The difference has been tacitly attributed to the presence of uricase in most mammals and its absence in man, the anthropoid ape, and the Dalmatian dog. Two recent studies from different laboratories have demonstrated that the anomaly must be explained by something other than the presence or lack of the uricase system. Young, Conway & Crandall (130) have conducted well controlled feeding experiments with Dalmatian puppies. It is found that the uricolytic index, varying from 29 to 90, is characteristic of both the diet and the individual dog. As supplements to the basal diet, both histidine and arginine yield slight increases in the output of allantoin, but not of uric acid. Likewise, guanine, xanthine, uric acid, and nucleic acid increased the output of allantoin and urea. The excretion of uric acid was constant throughout the period of the

experiment. The suggestion is offered that uric acid is a product of endogenous metabolism. Klemperer, Trimble & Hastings (131) have found the liver of a Dalmatian dog to be rich in uricase, but no correlation between the amount of uricase and the destruction of uric acid *in vivo* could be found. It is suggested that the inactivity of uricase *in vivo* may be due to the inaccessibility of the site of the uricase in the cell to the urate ion, or that a chemical inactivation of the enzyme by the intact cell may obtain.

In continuation of earlier interest in the purine metabolism of the kidneys, Reindel & Schuler (132) find that slices of the kidneys of rats, guinea pigs, and cats produce uric acid aerobically from xanthine and not from hypoxanthine. The oxidation of hypoxanthine to uric acid proceeds as two independent reactions. The latter observation has been confirmed by Bigwood, Thomas & Herbo (133). Reindel & Schuler further point out that in systems that contain hypoxanthine and xanthine, the formation of uric acid does not begin until all of the hypoxanthine has been converted to xanthine. In systems that contain uric acid, hypoxanthine, and xanthine dehydrogenase, uric acid decreases at the beginning both aerobically and anaerobically. The reaction is believed to proceed by way of a correlated oxidation of hypoxanthine and a reduction of uric acid, both substances ultimately forming uric acid. Thus, it is claimed that uric acid, under certain conditions, can be converted to xanthine in the body.

Doubt concerning the type of purine found to be synthesized in pigeon liver slices has arisen. Reindel & Schuler (134) believe that pigeon liver slices synthesize xanthine from alanine and some source, as yet unknown, of carbon. It is their contention that no purine other than xanthine is produced. Also, since the slices are found not to produce the xanthine from either adenine or hypoxanthine, but do form it from guanine, only the latter can be an intermediate in the synthesis. Örström, Örström & Krebs (135) are at a loss to explain the foregoing results. In their experiments, the purine that is noted to be synthesized is quantitatively converted into uric acid with the utilization of one mole of oxygen. This would indicate that the purine thus synthesized is hypoxanthine. It is suggested that the xanthine found by Reindel & Schuler may have arisen hydrolytically from guanine. Örström, Örström & Krebs also find that the rate of synthesis of hypoxanthine is increased by the addition of glutamic and oxaloacetic acid. The interpretation of these data must await further experimental evidence.

In an effort to obtain more data regarding the types of nucleotides and nucleosides that may give rise to hypoxanthine or xanthine in the liver, Euler and Skarżyński (136) have used the method of Thunberg. It is found that extracts of rat and rabbit livers catabolize adenosine, adenosine-5-phosphoric acid, and hypoxanthine. No utilization of adenine or guanylic acid could be noted, although extracts of rat embryos were able to utilize guanylic acid.

LITERATURE CITED

1. FEULGEN, R., BEHRENS, M., AND MAHDIHASSAN, S., *Z. physiol. Chem.*, **246**, 203 (1937)
2. BEHRENS, M., *Z. physiol. Chem.*, **253**, 185 (1938)
3. DELAPORTE, B., AND ROUKHELMAN, N., *Compt. rend.*, **206**, 1399 (1938)
3a. GULLAND, J. M., *J. Chem. Soc.*, 1722 (1938); BREDERECK, H., *Fortschr. Chem. organ. Naturstoffe*, **1**, 121 (1938)
4. GULLAND, J. M., AND JACKSON, E. M., *J. Chem. Soc.*, 1492 (1938)
5. GULLAND, J. M., AND JACKSON, E. M., *J. Chem. Soc.*, 1842 (1939)
6. BREDERECK, H., AND RICHTER, G., *Ber.*, **69**, 1129 (1936)
7. BREDERECK, H., KÖTHNIG, M., AND LEHMANN, G., *Ber.*, **71**, 2613 (1938)
8. TIPSON, R. S., AND LEVENE, P. A., *J. Biol. Chem.*, **127**, 105 (1939)
9. FALCONER, R., GULLAND, J. M., HOBDAY, G. I., AND JACKSON, E. M., *J. Chem. Soc.*, 907 (1939)
10. GULLAND, J. M., *Chemistry & Industry*, 321 (1940)
11. BREDERECK, H., CARO, G., AND RICHTER, F., *Ber.*, **71**, 2389 (1938)
12. BREDERECK, H., AND MÜLLER, G., *Ber.*, **72**, 1429 (1939)
13. LEVENE, P. A., AND JACOBS, W. A., *J. Biol. Chem.*, **12**, 411 (1912)
14. THANNHAUSER, S. J., AND OTTENSTEIN, B., *Z. physiol. Chem.*, **114**, 17 (1921)
15. BREDERECK, H., AND CARO, G., *Z. physiol. Chem.*, **253**, 170 (1938)
16. LEVENE, P. A., *J. Biol. Chem.*, **126**, 63 (1938)
17. BREDERECK, H., AND MÜLLER, G., *Ber.*, **72**, 115 (1939)
18. SIGNER, R., CASPERSSON, T., AND HAMMARSTEN, E., *Nature*, **141**, 122 (1938)
19. ASTBURY, W. T., AND BELL, F. O., *Nature*, **141**, 747 (1938)
20. SCHMIDT, G., AND LEVENE, P. A., *Science*, **88**, 172 (1938)
21. SCHMIDT, G., PICKELS, E. G., AND LEVENE, P. A., *J. Biol. Chem.*, **127**, 251 (1939)
22. HAMMARSTEN, E., *Biochem. Z.*, **144**, 383 (1924)
23. NEWMANN, A., *Arch. Anat. Physiol., Physiol. Abt.*, 374 (1898); Suppl. 552 (1899)
24. FEULGEN, R., *Z. physiol. Chem.*, **90**, 261 (1914)
25. LEVENE, P. A., *J. Biol. Chem.*, **53**, 441 (1922)
26. FEULGEN, R., *Z. physiol. Chem.*, **238**, 105 (1936)
27. JONES, W., *Am. J. Physiol.*, **52**, 203 (1920)
28. DUBOS, R. J., *Science*, **85**, 549 (1937); *J. Exptl. Med.*, **65**, 873 (1937)
29. DUBOS, R. J., AND THOMPSON, R. H. S., *J. Biol. Chem.*, **124**, 501 (1938)
30. SCHMIDT, G., AND LEVENE, P. A., *J. Biol. Chem.*, **126**, 423 (1938)
31. KUNITZ, M., *Science*, **90**, 112 (1939); *J. Gen. Physiol.*, **24**, 15 (1940)
32. ALLEN, F. W., AND EILER, J. J., *J. Biol. Chem.*, **137**, 757 (1941)
33. LEVENE, P. A., AND SIMMS, H. S., *J. Biol. Chem.*, **70**, 327 (1926)
34. MAKINO, K., *Z. physiol. Chem.*, **236**, 201 (1935)
35. BAKER, GULLAND, J. M., AND PRIDEAUX (Unpublished work); cited in GULLAND, J. M., *J. Chem. Soc.*, 1722 (1938)
36. TAKAHASHI, H., *J. Biochem. (Japan)*, **16**, 463 (1932)
37. BREDERECK, H., AND KÖTHNIG, M., *Ber.*, **72**, 121 (1939)
38. TIPSON, R. S., *J. Biol. Chem.*, **120**, 621 (1937)

39. Wagner-Juaregg, T., *Z. physiol. Chem.,* **239,** 188 (1936)
40. Loring, H. S., *J. Biol. Chem.,* **130,** 251 (1939)
41. Falconer, R., and Gulland, J. M., *J. Chem. Soc.,* 1912 (1937)
42. Gulland, J. M., and Story, L. F., *J. Chem. Soc.,* 259 (1938)
43. Gulland, J. M., and Story, L. F., *J. Chem. Soc.,* 692 (1938)
44. Stevens, C. D., *J. Biol. Chem.,* **120,** 751 (1937)
45. Semmens, C. S., *Nature,* **146,** 130 (1940)
46. Barber, H. N., and Price, J. R., *Nature,* **146,** 335 (1940)
47. Stenhagen, E., and Teorell, T., *Nature,* **141,** 415 (1938); *Trans. Faraday Soc.,* **35,** 743 (1939)
48. Mazza, F. P., and Tappi, G., *Arch. sci. biol. (Italy),* **24,** 526 (1938)
49. Lavin, G. I., Loring, H. S., and Stanley, W. M., *J. Biol. Chem.,* **130,** 259 (1939)
50. Koyenuma, N., *Biochem. Z.,* **304,** 332 (1940)
51. Greenstein, J. P., and Jenrette, W. V., *J. Natl. Cancer Inst.,* **1,** 77 (1940)
52. Hunter, G., and Hlynka, I., *Biochem. J.,* **31,** 486 (1937)
53. Bredereck, H., and Rothe, G., *Ber.,* **71,** 408 (1938)
54. Levene, P. A., and La Forge, F. B., *Ber.,* **45,** 608 (1912)
55. Bredereck, H., *Ber.,* **71,** 1013 (1938)
56. Hartmann, M., and Bosshard, W., *Helv. Chim. Acta,* **21,** 1554 (1938)
57. Klein, W., *Z. physiol. Chem.,* **255,** 82 (1938)
58. Ostern, P., and Terszakoweć, J., *Z. physiol. Chem.,* **250,** 155 (1937); Ostern, P., Baranowski, T., and Terszakoweć, J., *Z. physiol. Chem.,* **251,** 258 (1938); Ostern, P., Terszakoweć, J., and Hubl, S., *Z. physiol. Chem.,* **255,** 104 (1938)
59. Bredereck, H., and Richter, G., *Ber.,* **71,** 718 (1938)
60. Jachimowicz, T., *Biochem. Z.,* **292,** 356 (1937)
61. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.,* **121,** 131 (1937)
62. Bredereck, H., Berger, E., and Ehrenberg, J., *Ber.,* **73,** 269 (1940)
63. Gulland, J. M., and Hobday, G. I., *J. Chem. Soc.,* 746 (1940)
64. Euler, H. v., and Schlenk, F., *Z. physiol. Chem.,* **246,** 64 (1937)
65. Schlenk, F., Euler, H. v., Heiwinkel, H., Gleim, W., and Nyström, H., *Z. physiol. Chem.,* **247,** 23 (1937)
66. Vestin, R., and Euler, H. v., *Z. physiol. Chem.,* **247,** 43 (1937)
67. Vestin, R., Schlenk, F., and Euler, H. v., *Ber.,* **70,** 1369 (1937)
68. Schlenk, F., *Naturwissenschaften,* **25,** 270 (1937)
69. Das, N. B., and Euler, H. v., *Nature,* **141,** 604 (1938)
70. Schlenk, F., *Naturwissenschaften,* **28,** 46 (1940)
70a. Baumann, C. A., and Stare, F. J., *Physiol. Rev.,* **19,** 353 (1939); Lutwak-Mann, C., *Biol. Rev.,* **14,** 399 (1939)
71. Meyerhof, O., and Ohlmeyer, P., *Biochem. Z.,* **290,** 334 (1937)
72. Ochoa, S., *Biochem. Z.,* **292,** 68 (1937)
73. Klein, J. R., *J. Biol. Chem.,* **134,** 43 (1940)
74. Williamson, S., and Green, D. E., *J. Biol. Chem.,* **135,** 345 (1940)
75. Warburg, O., and Christian, W., *Biochem. Z.,* **287,** 291 (1936)
76. Euler, H. v., and Bauer, E., *Ber.,* **71,** 411 (1938)
77. Euler, H. v., and Adler, E., *Z. physiol. Chem.,* **252,** 41 (1938)

78. LOHMANN, K., AND SCHUSTER, P., *Naturwissenschaften,* **25,** 26 (1937) ; *Biochem. Z.,* **294,** 188 (1937)
79. HENNESSY, D. J., AND CERECEDO, L. R., *J. Am. Chem. Soc.,* **61,** 179 (1939)
80. EULER, H. V., AND VESTIN, R., *Naturwissenschaften,* **25,** 416 (1937)
81. TAUBER, H., *Science,* **86,** 180 (1937) ; *Enzymologia,* **2,** 171 (1937)
82. OCHOA, S., *Biochem. J.,* **33,** 1262 (1939)
83. STERN, K. G., AND HOFER, J. W., *Enzymologia,* **3,** 82 (1937)
84. TAUBER, H., *J. Am. Chem. Soc.,* **60,** 730 (1938) ; *J. Biol. Chem.,* **125,** 191 (1938)
85. KREBS, H. A., *Klin. Wochschr.,* **11,** 1744 (1932) ; *Z. physiol. Chem.,* **217,** 191 (1933)
86. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.,* **298,** 150 (1938)
87. BALL, E. G., *Science,* **88,** 131 (1938) ; *J. Biol. Chem.,* **128,** 51 (1939)
88. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.,* **298,** 368 (1938)
89. HAAS, E., *Biochem. Z.,* **298,** 378 (1938)
90. CORRAN, H. S., GREEN, D. E., AND STRAUB, F. B., *Biochem. J.,* **33,** 793 (1939)
91. KLEIN, J. R., AND KOHN, H. I., *J. Biol. Chem.,* **136,** 177 (1940)
92. KIESSLING, W., AND MEYERHOF, O., *Naturwissenschaften,* **26,** 13 (1938) ; *Biochem. Z.,* **296,** 410 (1938)
93. DAVIS, A. R., NEWTON, E. B., AND BENEDICT, S. R., *J. Biol. Chem.,* **54,** 595 (1922)
94. FALCONER, R., AND GULLAND, J. M., *J. Chem. Soc.,* 1369 (1939)
95. BARRENSCHEEN, H. K., AND JACHIMOWICZ, T., *Biochem. Z.,* **292,** 350 (1937)
96. DILLER, I. M., *J. Biol. Chem.,* **118,** 161 (1937)
97. NEWTON, E. B., *J. Biol. Chem.,* **120,** 315 (1937)
98. FLORKIN, M., *Arch. intern. physiol.,* **44,** 542 (1937)
99. BRØCHNER-MORTENSEN, K., *Acta Med. Scand.,* Suppl., **84,** 269 pp. (1937)
100. BRØCHNER-MORTENSEN, K., *Medicine,* **19,** 161 (1940)
101. BLAUCH, M. B., AND KOCH, F. C., *Proc. Soc. Exptl. Biol. Med.,* **38,** 638 (1938) ; *J. Biol. Chem.,* **130,** 443 (1939)
102. BOSSON, G., *Mikrochim. Acta,* **2,** 74 (1937)
103. PAGET, M., AND BERGER, R., *Compt. rend. soc. biol.,* **127,** 1312 (1938)
104. FLORKIN, M., AND BOSSON, G., *Bull. soc. chim. biol.,* **21,** 673 (1939)
105. SCHAFFER, C. F., AND GREENBAUM, F. R., *J. Lab. Clin. Med.,* **25,** 1206 (1940)
106. PARNAS, J. K., AND UMSCHWEIF, B., *Bull. soc. chim. biol.,* **19,** 325 (1937)
107. PEHAM, A., *Mikrochim. Acta,* **2,** 65 (1937)
108. KERR, S. E., AND BLISH, M. E., *J. Biol. Chem.,* **98,** 193 (1932)
109. KERR, S. E., AND SERAIDARIAN, K., *J. Biol Chem.,* **132,** 147 (1940)
110. ALLEN, F. W., LUCIA, S. P., AND EILER, J. J., *Proc. Soc. Exptl. Biol. Med.,* **34,** 609 (1936)
111. EILER, J. J., AND ALLEN, F. W., *J. Biol. Chem.,* **123,** 655 (1938)
111a. ALLEN, F. W. (Unpublished observation)
112. MEJBAUM, W., *Z. physiol. Chem.,* **258,** 117 (1939)
113. PARNAS, J. K., AND LUTWAK-MANN, C., *Biochem. Z.,* **278,** 11 (1935)
114. CONWAY, E. J., AND COOKE, R., *Biochem. J.,* **33,** 464 (1939)

115. BORSOOK, H., AND DUBNOFF, J. W., *J. Biol. Chem.*, **131**, 163 (1939)

116. RAEKALLIO, T., *Skand. Arch. Physiol.*, **81**, 1 (1939)

117. KREBS, H. A., AND ÖRSTRÖM, Å., *Biochem. J.*, **33**, 984 (1939)

118. MOZOLOWSKI, W., *Biochem. Z.*, **206**, 150 (1929)

119. HELLER, J., AND KLISIECKI, A. J., *Biochem. Z.*, **275**, 362 (1935)

120. CONWAY, E. J., AND COOKE, R., *Nature*, **139**, 627 (1937) ; **142**, 720 (1938) ; *Biochem. J.*, **33**, 457, 479 (1939)

121. DRURY, A. N., LUTWAK-MANN, C., AND SOLANDT, O. M., *Quart. J. Exptl. Physiol.*, **27**, 215 (1938)

122. KERR, S. E., AND ANTAKI, A., *J. Biol. Chem.*, **121**, 531 (1937)

123. BLAUCH, M. B., AND KOCH, F. C., *J. Biol. Chem.*, **130**, 455 (1939)

124. BLAUCH, M. B., KOCH, F. C., AND HANKE, M. E., *J. Biol. Chem.*, **130**, 471 (1939)

125. REDING, R., *Compt. rend. soc. biol.*, **126**, 95 (1937)

126. OPSAHL, R., *Acta Med. Scand.*, **102**, 611 (1939)

127. BRUNEL, A., *Compt. rend.*, **204**, 380 (1937)

128. DAVIDSON, J. N., *Nature*, **141**, 790 (1938) ; *Biochem. J.*, **32**, 1386 (1938)

129. HOLMBERG, C. G., *Nature*, **143**, 604 (1939)

130. YOUNG, E. G., CONWAY, C. F., AND CRANDALL, W. A., *Biochem. J.*, **32**, 1138 (1938)

131. KLEMPERER, F. W., TRIMBLE, H. C., AND HASTINGS, A. B., *J. Biol. Chem.*, **125**, 445 (1938)

132. REINDEL, W., AND SCHULER, W., *Z. physiol. Chem.*, **247**, 172 (1937)

133. BIGWOOD, E. J., THOMAS, J., AND HERBO, H., *Compt. rend. soc. biol.*, **126**, 913 (1937)

134. REINDEL, W., AND SCHULER, W., *Z. physiol. Chem.*, **248**, 197 (1937)

135. ÖRSTRÖM, Å., ÖRSTRÖM, M., AND KREBS, H. A., *Biochem. J.*, **33**, 990 (1939)

136. EULER, H. v., AND SKARŻYŃSKI, B., *Z. physiol. Chem.*, **263**, 259 (1940)

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THE BIOCHEMISTRY OF CREATINE AND CREATININE[1]

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Methods. Distribution of true creatine and creatinine.—The chemistry of the Jaffe reaction with alkaline picrate has been studied by Bolliger (1, 2, 3). When different amounts of sodium hydroxide are added to an alcoholic solution of creatinine and picric acid four compounds are formed: the first contains 1 mol of picric acid and 2 mols of sodium hydroxide; the second, third, and fourth contain respectively, 2½, 3, and 3½ mols of sodium hydroxide for each mol of creatinine. Compound 4 represents the maximum degree of saturation with alkali and was considered by Bolliger to be most responsible for the Jaffe reaction.

The lack of specificity of this reaction has been criticized ever since it was first introduced by Jaffe in 1886. Although it is true that many compounds give the reaction *in vitro,* it is equally true that about 98 per cent of these compounds are not present in body tissues and fluids. It has long been supposed that a chromogenic substance is present in blood which is not creatinine. In 1927 Gaebler & Keltch (4) stated that both normal and retention blood samples contained chromogenic substances other than creatinine; the same view was emphasized by Bohn & Hahn (5). Matsumoto (6) boiled pig's brain with absolute alcohol and extracted the residue with ether and finally with water. The majority of the creatine was found in the alcoholic and water extracts while the ether extract contained about 14 per cent of the total creatinine of the brain. Since creatine itself is insoluble in ether it was concluded that the previously reported values for brain creatine were too high.

Miller & Dubos (7, 8, 9) described methods for the specific enzymatic determination of creatine and creatinine in body fluids. These

[1] The space allotted for this review, which covers the literature during the last ten years or more up to December 20, 1940, will permit a discussion of only those advances which, as suggested by the Editors, the reviewer considers will be of most help in evaluating the present status of the several divisions of the subject discussed. In a subsequent review, to be published elsewhere, a number of remaining topics will be considered.

methods have proved of much value in elucidating several perplexing problems in creatine and creatinine metabolism. Determinations were made of the true creatinine content of the plasma, serum, red cells, and urine of normal individuals and of those suffering from various degrees of diminished kidney function. It was observed that true creatinine constitutes from 80 to 100 per cent of the chromogenic material in serum and plasma and from 30 to 50 per cent in the red cells. In urine of normal and nephritic individuals creatinine constituted nearly 100 per cent of the chromogenic material present.

Miller *et al.* (10) also published a specific enzymatic method for the determination of creatine and creatinine in tissues. Baker & Miller (11), using this method, showed that practically all of the chromogenic material in muscle filtrates was true creatine. It is therefore no longer necessary to use the specific enzymatic method routinely in the determination of muscle creatine and of urinary creatine and creatinine. Sullivan & Irreverre (12) reported a new and highly specific reaction for creatinine, in which 1,4-naphthoquinone-2-potassium sulfonate is employed.

In skeletal and heart muscle, testis, and brain the true creatine averages from 83 to 96 per cent of the apparent creatine but, in other tissues, such as the intestinal muscle, pancreas, spleen, lung, and liver, the true creatine amounts to only 16 to 65 per cent of the apparent value (11). It was also found that the concentration of creatinine was highest in muscle, testis, and brain, and considerably lower in kidney, pancreas, lung, and liver. The range was from 0.1 to 4.8 mg. per 100 gm. of tissue. The concentrations of creatine and creatinine were roughly proportional in the different tissues, indicating an interrelationship of these two compounds in metabolism. The concentration of creatinine in the spleen, lung, liver, and pancreas was much lower than in an ultrafiltrate of plasma; this suggests that these tissues are concerned with the conversion of creatinine into creatine or other compounds.

It should be remembered that bacteria produce the so-called "adaptive" enzymes on any media containing carbon and nitrogen, or if ammonium carbonate be present, on other media which contain carbon compounds, such as glucose. It is therefore necessary to grow the cultures on creatinine if the suspensions obtained are to be used in the determination of creatine and creatinine. The interesting finding was made by Miller & Baker (13) that these suspensions will also oxidize sarcosine, a fact which suggests that sarcosine is an intermediate in

the oxidation of creatinine. Dubos (14) has recently reviewed the literature on the adaptive production of enzymes by bacteria. The specificity of the creatinine oxidases has been determined by testing them against a number of substrates related to creatinine. The addition of a methyl or acetyl group to the creatinine molecule completely inhibits the action of the enzymes; the transfer of the methyl group in position 3 (leaving glycocyamidine) to position 5 considerably retards the action of one of the enzymes and completely inhibits the action of the other. The enzyme which converts creatine to creatinine shows a similar specificity (15). This adaptive anhydrase offers an interesting example of the enzymatic combination of an amino and a carboxyl group to form the CO-NH linkage, and this reaction may be useful in studying the metabolism of creatine and creatinine. It is also interesting that this enzyme was formed equally well when creatinine instead of creatine was added to the medium.

The nature of the "residual chromogen" (the colored compound formed in the Jaffe reaction which is not destroyed by the creatinine enzyme of Miller & Dubos) of body tissues and fluids has not been determined. Gerard & Tupikova (16) showed that in resting oxygenated tissue the total creatine was partitioned between phosphocreatine, free creatine, and residual chromogen in the following percentages: nerve, 40, 40, 20; muscle, 50, 44, 4; and brain, 20, 60, 20. They believe that the residual chromogen is not creatine and that it may be bound to protein. Myers & Mangun (17) observed a correlation between the amounts of phosphorus, potassium, and creatine in brain and in various muscles, which suggests the possibility that creatine may exist as the dipotassium salt of creatine phosphoric acid. Myers & Mangun (18) reviewed the relation of creatine to the heart in health and disease.

Some investigators in the past have denied the existence of creatinine in the blood. The final answer to this problem is evidently furnished by the studies of Miller & Dubos (8) who, as stated above, showed that from 80 to 100 per cent of the chromogenic material of serum and plasma is true creatinine. Plasma from uremic patients, however, contained a large amount of nonchromogenic material which in several patients was proportional to the severity of the uremic toxemia.

At the present time, with the introduction of spectrophotometric methods for measuring color intensities and with the use of the specific creatinine enzymes of Miller & Dubos, the student of creatine and

creatinine metabolism can have a much higher degree of confidence in his results than heretofore, and criticisms of the lack of specificity of the Jaffe reaction for creatinine in some body tissues (muscle) and fluids (plasma and urine) are no longer justified. The reviewer predicts that with the modifications discussed above, this reaction as applied by Folin in 1905 will remain the standard method for the determination of creatine and creatinine for many years to come.

The origin of creatine.—Probably no subject of investigation in metabolism has been more debated than has the origin of creatine. Even though it is obvious that the nitrogen of creatine must originate from that of the amino acids, many negative results with these substances as creatine precursors in the past have served to cast doubt on this hypothesis. These statements also apply to the origin of creatinine. While several criticisms of the experimental conditions employed by many investigators could be offered here, suffice it to say that two of the most important of those necessary for creatine formation were not always present: (*a*) a normal protein diet, and (*b*) sufficient amounts of the amino acids fed or injected to the experimental animals or man. When these two conditions are fulfilled, it is easy to believe that the amino acids are the most logical precursors of both creatine and creatinine, as the following discussion will show.

Beard *et al.* (19 to 48) have submitted seven reactions to illustrate the synthesis of creatine and creatinine *in vivo*. These reactions postulate that glycine, sarcosine, arginine, and methylamine may serve as precursors. In certain cases urea and, possibly, cyanic acid participate in the synthesis. Depending upon the reaction in question, hydantoic acid, methyl hydantoic acid, or guanidine butyric acid may serve as an intermediate. The formation of creatine from glycine and urea, from sarcosine and urea, from arginine, and from creatinine was demonstrated under their experimental conditions. The other reactions are of hypothetical interest only.

The results of Beard *et al.* may be summarized as follows:

(*a*) Feeding of proteins or amino acids, except proline and oxyproline, or injection of amino acids and related substances, increases the concentration of muscle creatine in the rat (19, 20, 26, 27, 28, 29, 39, 44) and the dog (25) and creatine-creatinine excretion in the rat (19, 26, 28, 29) and man (21, 22, 23, 24, 28, 29). (*b*) Creatine formation under these conditions is not due to the specific dynamic action of the amino acids (19). (*c*) The amino acids may be metabolized through glycine and urea in creatine synthesis (27). (*d*) The presence of the amino group of the amino acid is necessary for creatine formation (19, 27). (*e*) The guanidine group of creatine is synthetic in origin and can arise from

arginine, urea, creatinine, glycocyamine, or glycocyamidine by the process of transamidination (19, 21, 26, 27, 28, 29). (*f*) Increasing the methylation process in the body results in increased creatine formation (30, 31, 32). (*g*) Creatine is usually formed from the proteins and amino acids and its synthesis need not be limited to the physiological requirement for it (27). (*h*) Creatine storage in the muscles of the rat (19, 20, 27) and probably also in man (48) is of short duration (two to four days). (*i*) Injection of urea with glycine, sarcosine, betaine, or choline results in more than twice as much extra creatine in young rats as the injection of the same amounts of these substances without urea (27). (*j*) Urea and glycine, used in the formation of creatine and creatinine, can each be synthesized in the rat (28). (*k*) Amino acids may also serve as sources of urea in this connection (27, 28). (*l*) Glycine peptides and glutathione may act as sources of glycine, and hydantoin may supply both urea and glycine (27). (*m*) The extent of creatine formation probably depends upon the amount of available phosphate present in the tissues (38, 48). (*n*) Injection of small doses of the purines and their methylated derivatives (31, 32), or of 1 gm. of creatinine (33), greatly stimulates the excretion of both creatine and creatinine. (*o*) The injection of creatinine and water (or physiological saline) together gives twice as much extra creatine excretion as the injection of either alone. The same was true in the case of water *vs.* saline (38). (*p*) The body metabolizes administered creatine and creatinine in a manner different from that in which it utilizes creatine and creatinine formed in the course of normal amino acid metabolism. Administered creatine is of little value to the myopathic patient and is largely excreted as such (21, 22, 23, 24). It does not increase the energy output in students when ingested (48). However, creatine formed from the amino acids or creatinine during amino acid metabolism is changed into phosphocreatine, which plays an important part in supplying energy for muscular contraction (43, 48). The ingestion of glycine and urea together gave over twice as great an energy output in men and women as that obtained from the ingestion of the same amount of glycine alone (43). Also the energy output in students after the ingestion of glycine with urea (wine or tablets) or glycine (wine or tablets) was directly proportional to the amounts of these substances ingested daily (48). Increases in energy output were first noticeable in about three weeks after ingestion of these substances. On the other hand, this increased energy output was rapidly lost in three or four days after their ingestion was discontinued.

Schoenheimer *et al.* (49 to 56, 58) have made very important contributions to the subject of the origin of creatine. Ammonia containing N^{15} was utilized by the rat and was, in part, transformed into creatine containing N^{15} (49, 50). Glycine, and other amino acids were shown to donate and accept nitrogen (transamination) from each other in protein metabolism (51). Several amino acids and related substances, *dl*-tyrosine, *l*-leucine, sarcosine, glycine, arginine, glycocyamine, and urea, each containing N^{15} were transformed into creatine containing N^{15} (52, 53, 54, 55). After feeding arginine containing N^{15} a large amount of creatine containing N^{15} was isolated,

showing that the amidine group of creatine must have arisen from the amidine group of arginine (transamidination) which has recently been shown by Schoenheimer and his associates to be continuously formed in normal animals from the α-amino nitrogen of various amino acids.

The nitrogen of the amidine group of creatine was also obtainable from other amino acids, while glycine or sarcosine served to increase the glycine moiety of creatine (49). The ingestion of sarcosine containing N^{15} led to the deposition in proteins of glycine containing N^{15} to the same extent as when glycine was fed. It was concluded, therefore, that sarcosine does not appear to be an intermediate in creatine formation but leads to its production through demethylation to glycine (56), a reaction also observed by Abbott & Lewis (57). The administration of creatine containing N^{15} resulted in the formation of phosphocreatine containing N^{15} in muscle and internal organs (58). Only 2 per cent of body creatine of adult animals was formed per day, which was about equal to the creatinine excretion during this time.[2] Creatine containing N^{15} was not destroyed, since there was no increase in ammonia or urea.

The reader is no doubt bewildered by the large number of different substances that are able to increase creatine formation and excretion. In considering the large number of metabolic changes which may occur in feeding and injection experiments, many direct and indirect effects on creatine formation are possible from substances which supply urea, arginine, creatinine, or glycocyamine for the guanidine group of creatine; and glycine or methionine or other amino acids for the methyl group. The discovery of reactions of amination, deamination, transamination, transamidination, and transmethylation, by Braunstein & Kritsmann (59), Schoenheimer *et al.* (49 to 56, 58), Borsook & Dubnoff (60, 61) and du Vigneaud *et al.* (62, 63) must also be considered in this connection. Practically any amino acid except proline and oxyproline, provided it be fed or injected in sufficient amounts, can serve to increase creatine formation and excretion (27). Thomas (64) and Rose (65) expressed the view that exogenous arginine was not the mother substance of creatine or creatinine, but believed that tissue arginine may be an intermediate in its formation. The above

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[2] These deductions of Bloch & Schoenheimer may apply to the rat but they probably do not apply to man. If it be assumed that a man weighing 70 kg. contains about 140 gm. of creatine in his muscles (171), then he should excrete 2 per cent, or 2.8 gm. of this daily as creatinine. It is well known that the average male adult fed on a normal protein diet excretes, on the average, from 1.5 to 1.7 gm. of creatinine daily (46).

evidence, however, gives definite proof that the amino acids, no matter what their source, are to be considered as the normal precursors of both creatine and creatinine in the body.

After Beard & Pizzolato (27) published their urea-glycine scheme for creatine formation, Fisher & Wilhelmi (66) offered several theoretical criticisms, none of which is acceptable to Beard & Pizzolato.

Studies have been reported by a number of workers on the formation of creatine *in vitro:* by Borsook & Dubnoff (60, 61) from glycocyamine; by Baker & Miller (67) using isolated rat tissues; by Shibuya (68) from arginine with glycine and thyroxine; by Shibuya (69) from glycine with glycocyamine; by Sasaki (70) from urea with glycine; by Beard & Espenan (37) from urea with glycine and glycine with cyanamide (to form glycocyamine) and from sarcosine with urea and sarcosine with cyanamide (the cyanamide studies were conducted with and without the addition of ammonia); by Bach (71) on heart tissue and glycocyamine; by Fisher & Wilhelmi (72) from glycocyamine using the perfused heart; and by Davenport *et al.* (73) in heart perfusion experiments on glycocyamine and glycollic acid.

Fitzgerald & Schmidt (74) showed many years ago that bacteria produced creatinine, and Sears (75, 76) reported that creatinine was formed in considerable quantities from peptone by *Proteus vulgaris.* Fish & Beckwith (77) also observed that creatinine was formed in large amounts from several bacterial species. The participation of urea and glycine in creatinine synthesis was shown. The destruction of the guanidine portion of arginine with resultant formation of urea gave rise to creatinine under the enzymatic influence of *Proteus vulgaris.* The yield from highest to lowest was as follows: cystine, glycine, aspartic acid, *dl*-tyrosine, *dl*-leucine, *d*-arginine, *dl*-alanine, phenylalanine, and *dl*-glutamic acid.

Much interest has recently been shown in the nature of the methylating agent in creatine formation. Stekol & Schmidt (78) were the first to show that *dl*-methionine increased creatinine excretion in the dog. Beard & Pizzolato (27) showed that the injection of methionine increased creatine formation in young rats about 25 per cent, while methyl methionine increased it over 100 per cent. Kelly & Beard (30) observed similar increases in creatine excretion after the injection of methyl urea, as compared to urea. Borsook & Dubnoff (60, 61) showed that glycocyamine was methylated by methionine to creatine in the presence of liver slices from various animals. Creatine formation from arginine and glycine was rapid (transamidination). Arginine and sarcosine yielded glycocyamine and not creatine. This

indicates that sarcosine was demethylated to glycine in creatine synthesis. Bloch & Schoenheimer (56) and Abbott & Lewis (57) also showed that this demethylation occurs in the rat. Du Vigneaud *et al.* (62, 63) marked the methyl group of methionine with deuterium and obtained definite evidence of the shift of this group to creatine and choline. According to Bloch & Schoenheimer (50) this finding, in conjunction with the results of Borsook & Dubnoff (60, 61) and their own (49, 56, 58), establishes the biological origin of all parts of the creatine molecule. The carbon chain and one nitrogen atom are derived from glycine, the amidine group from arginine and the methyl group from methionine.[8]

This theory is too specific. If the view be accepted that sarcosine serves in creatine formation only as a precursor of glycine, then it must be assumed that equivalent amounts of sarcosine and glycine would result in about the same increases in creatine formation and excretion. This is certainly not the case. In studies of Beard *et al.* sarcosine always gave more creatine formation and excretion than did equivalent amounts of glycine. This increase above that obtained from glycine is equivalent to 15 or 20 per cent, which is due to the methyl group of sarcosine alone (27, 29, 30, 31, 32). It is therefore established that both the methyl group and glycine moiety of sarcosine take part in creatine synthesis when sarcosine is administered. The view that glycine, arginine, glycocyamine, and methionine are the only substances that take part in the biological synthesis of creatine is also too specific. They may be the most important ones in this connection but there are many other substances, as the following discussion will show, that increase creatine and creatinine formation and excretion.

Hoppe-Seyler (80) showed in 1930 that glycine acted as a methylating agent. Challenger (81) pointed out that biological methylation in microorganisms, plants, and animals may take place through the action of this amino acid. Through its deamination products, glyoxylic acid and formaldehyde, it is capable of methylating other amino acids, including itself, to yield sarcosine and betaine. [Fosse & de Larambergue (82, 83) showed that cyanamide is formed *in vitro* by the oxidation of sugars in the presence of ammonia and that formaldehyde

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[8] Griffith & Mulford (79) showed that creatine decreases somewhat the renal lesions, hemorrhagic degeneration, renal hemorrhage, and fatty livers in young rats fed on diets low in choline. The methyl group of creatine is not available for the synthesis of choline. Dietary creatine, however, does spare the labile methyl supply and makes available for choline formation those methyl groups which would otherwise be used in the synthesis of creatine in the body.

was an intermediate product of the reaction.] Tripoli & Beard (21) suggested that glycine, as well as other amino acids, could supply the methyl group necessary for creatine formation. The same view was expressed by Mourot (84) and Bach (71).

Kelly & Beard (30) showed that compounds containing methyl groups, which, however, are known not to be methylating agents, do not influence creatine formation. Those compounds containing a methyl group attached to nitrogen, such as is found in the methyl amines, methyl urea, mecholyl, caffein, and other purines and related substances, increased creatine formation and excretion. Methylating agents, such as methyl alcohol, glycine, glycollic acid, and paraformaldehyde, did likewise. Glycine, by hydrolytic deamination, may be converted into glycollic acid, which may then act as the methylating agent in creatine formation. Davenport *et al.* (73) have shown that glycocyamine and glycollic acid increase the total creatinine content of isolated perfused rabbit hearts and that glycollic acid acts as a methylating agent in this connection. Milhorat & Toscani (85) observed that glycollic acid caused a slight increase in creatine excretion in their myopathic patients. Beard (25) showed that the injection of arginine or glycollic acid in the hepatectomized dog caused an increase in creatine formation in the muscles eight hours after injection. From these results it would appear that glycine and other substances may serve as methylating agents in creatine formation.

The purines and their methylated derivatives also play a very significant part in this methylating process. It is established from the earlier work of Abderhalden & Buadze (86, 87, 88), Chrometzka (89), and Zwarenstein (90), that purines, methylated purines, nuclear material, uric acid, hydantoin, and methyl hydantoin are precursors of creatine. Beard & Pizzolato (31) and Koven & Beard (32) showed that creatine formation in the muscles of rats and excretion in the urine were directly proportional to the number of methyl groups, up to three, present in the supplement injected [xanthine, 59 per cent; dimethylxanthine (theophyllin), 80 per cent; trimethylxanthine (caffeine), 99 per cent]. One methyl group was therefore again equivalent to an increase of about 20 per cent in creatine excretion. Other purines and uric acid also increased creatine formation and excretion.

The function of creatine.—The hydrolysis of phosphocreatine in muscular contraction is becoming clearer. According to Vague & Dunan (91), for muscular contraction there must be an equilibrium between the different phosphorized compounds and the successive agents of their hydrolysis and rephosphorylation: a balance of glyco-

gen, creatine, adenylic acid, and oxygen in a perfect acid-base equilibrium. At the present time it is believed that the hydrolysis of adenosinetriphosphate gives the immediate energy for muscular contraction; the hydrolysis of phosphocreatine, the energy for the resynthesis of adenosinetriphosphate; and the breakdown of glycogen to lactic acid, the energy for the resynthesis of phosphocreatine (92).[4]

Creatine is known to be an acceptor for phosphate during glycolysis. Sachs (94) has recently shown that the formation of creatine during muscular contraction also supplies base for the neutralization of some of the lactic acid formed. This confirms the view expressed many years ago by Fiske & Subbarow (95). Chaikoff *et al.* (96) showed that creatine does not stimulate phospholipid turnover in the liver. Almquist & Mecchi (97) have reported that creatine increases the growth of the chick more than does glycine or acetates. In the absence of glycine the chick develops muscular dystrophy.[5] Creatine and creatinine produce an increase in the amplitude of the beat of the perfused heart of *Xenopus laevis,* the rabbit, and the cat (98). They also produce a slight dilatation of the blood vessels in pithed toads. They have no effect on blood pressure in the cat.

Creatine-Creatinine Retention and Excretion. The Origin of Creatinine.—Beard & Jacob (33) reported that creatine retention varied from 20 to 76 per cent when doses from 25 to 200 mg. of creatine were injected. The creatinine retention, in relation to the dose of creatine injected, varied from 300 per cent for the 25 mg. dose to 32 per cent for the 200 mg. dose. In man even more striking results were obtained. Ingestion of 3 to 5 gm. of creatine by six normal subjects caused a creatine retention which varied from 0 to 1.9 grams of the dose ingested, and a creatinine retention which varied from 0 to 3 grams. In none of these cases was creatine transformed into creatinine. When creatinine, in doses of from 10 to 200 mg., was injected into young rats, the creatinine retention varied from 10 to 124 mg., and was accompanied by an increased creatine excretion of 23 to 60 mg. The ingestion of 0.5 to 5 gm. of creatinine by the six normal

[4] It has also been maintained that the oxidation of the lactic acid supplies some of the energy for the resynthesis of part of the lactate to glycogen. According to Hastings *et al.* (93), experiments with radioactive carbon present in the carboxyl group of sodium lactate, show that most of the lactic acid is oxidized to carbon dioxide and only a small part of it is resynthesized into glycogen.

[5] In a personal communication to the reviewer, Dr. Almquist stated that creatinine could likewise increase the growth of the chick to about the same extent as creatine. This is to be expected if creatinine were transformed into creatine in the body.

subjects again caused a retention of creatinine which varied from 0 to 3 gm. Creatinine may therefore be largely excreted or retained, depending upon the experimental subject. The increased creatine excretion under these conditions was striking, varying from 5 to 20 gm. The chromogenic material (Jaffe test) in all of the urines tested was destroyed by the creatinine enzyme of Miller & Dubos. The results of several other studies confirm and extend these findings (34, 35, 38, 39, 40).

These findings reveal a new feature of creatine and creatinine metabolism, namely, that the injection or ingestion of creatinine in the rat or man greatly stimulates the formation and excretion of creatine and creatinine. The creatinuria is not due to a liberation and excretion of muscle creatine or to changes in kidney function. So large were the increases in muscle creatine and in creatine excretion after the injection of very small doses of the purines and their methylated derivatives that Beard *et al.* (31, 32) concluded that a stimulation of creatine formation and excretion also occurred under these conditions. Schoo & Boer (99) stated that the spontaneously existing creatinuria in normal children is increased by giving them 1 gm. doses of creatinine.

Cameron (100) gave 1 gm. doses of creatinine to eight normal subjects. The blood creatinine content four hours later varied about 230 per cent. The normal concentration of blood creatine was also observed to vary from time to time. When a dose of 1 to 7 gm. of creatinine was ingested it was all excreted within one day. Espersen & Thomsen (101) concluded that the day-to-day variations in creatine and creatinine excretion were so great in cases of some of the myopathies that no conclusions could be drawn with respect to the action of glycine in these cases. This statement will hardly be accepted by students of creatine and creatinine metabolism.

When creatinine was injected into the rabbit, from 35 to 70 per cent was excreted into the urine and about 1 per cent in the bile (102). In uremia, Linegar, Frost & Myers (103) found an increase in heart- and skeletal-muscle creatine, phosphorus, and potassium, and stated that by retention of creatinine the equilibrium between creatine and creatinine would be shifted toward the former.

It has generally been believed that the normal adult male excretes little or no creatine. Djen & Platt (104) showed, however, that 108 out of 148 normal male Chinese subjects showed creatinuria and in many cases a remarkably low creatinine output. Hobson (105) found a mean value of 637 mg. of creatine per day (with a range of 92 to

1200 mg.) in ninety-seven normal male urine specimens. These were from students taking courses in physical training. When they were fed a diet low in carbohydrate the creatinuria disappeared. The creatinuria was believed to be due to the sugar feeding and not to the exercise. The increased Jaffe reaction was not due to the presence of sugar in the urine. These results confirm those of Haldi & Bachmann (106), who showed that glucose and fructose feeding results in creatinuria in man. Taylor & Chew (107) reported the excretion of 0 to 196 mg. of creatine per day in fifteen adult males. In any suspected case of creatinuria, the protein, sugar, and water intake should be known before conclusions are reached as to the relation of the creatinuria to the pathological condition of the patient.

Wang (108) reviewed the subject of creatinuria. Experimental work upon blood, urine, and muscles of animals and patients was conducted. Since creatinuria was produced under a variety of experimental and clinical conditions, he concluded that it was an entirely unspecific phenomenon. Thomasen (109) likewise reviewed the subject of creatinuria. The renal threshold for creatine was stated to be 1 to 1.25 mg. of creatine per 100 cc. of plasma. Creatine does not occur in the blood plasma unless it is ingested in the food. Breast milk contains 0.5 mg. and cow's milk 5 mg. of creatine per 100 cc. This may account for the low creatine excretion by breast-fed infants.

Mourot (84) made an extensive study of the nature of the excretory products resulting from the catabolism of amino acids in both young and old rats. Most of these, especially arginine, histidine, aspartic acid, and glycine, caused an increase in creatine excretion while creatinine excretion was not changed. She suggested that glycine might either methylate an intermediate product which contained the guanidine group and was formed from arginine or histidine, or might be directed from its normal path and combined with a precursor of urea [cyanic acid (110)] or with the tautomeric form of urea which Werner has shown to be active.

Folin (111) stated in 1905 that the creatinine output in man is independent of the protein intake. Even if this statement were true, it does not follow that the creatinine output will always be constant when increased amounts of the proteins or amino acids themselves are fed or injected. Boggess & Beard (26) observed increases up to 138 per cent in creatinine excretion in 60 per cent of rats injected with unusually large doses of glycine or arginine. Extra creatine excretion did not occur. The size of the dose of amino acid injected is important, small doses increasing creatine excretion and large ones increasing

creatinine excretion. These results confirm those of Beard & Barnes
(19) in which most of the proteins and amino acids ingested brought
about an increased elimination of creatinine by the rat and man.

The following substances have been shown to cause creatinuria
after their administration to animals or patients: glycine (112, 113,
114); glycine with phosphate (115); leucylglycine (116); glycollic
acid (85); alanine and leucine (117); tyrosine and histidine (118, 119,
120); arginine (121, 122); argininic acid (123); glucose and fructose
(105, 124); cholic acid (125, cf. 126); and iodoacetate (127). Crea-
tinuria was found also after exposure to x-rays (128) and low tem-
peratures (127, 129). It occurred after fractures (130) and after
blockade of the reticuloendothelial system (131, 132).

Increases in creatinine excretion have been reported after admin-
istration of the following substances: argininic acid (123); uric acid
(89, 90); histidine and nuclear material (86, 87, 88; and cholic acid
(133), though according to Taku (134) and Ikoma (135) injection
of bile acids into the rabbit causes a decrease in creatinine excretion.
Masai & Fukutomi (136) stated that administration of sarcosine, his-
tidine, urocanic acid, cystine, and taurine caused an increase in both
creatine and creatinine excretion.

Observations from Terroine's laboratory (137, 138, 139, 140)
show that creatine is synthesized from tissue proteins. Zamagi (141)
found that in fasting rats excretion of creatine was reduced while that
of creatinine was increased. Whipple et al. (142) showed that the
production of a sterile abscess in a dog caused large increases in
creatine and nitrogen excretion. Injection of dog plasma into a pro-
tein-depleted dog likewise caused an increase in creatine, ammonia,
and urea excretion. Chloroform anesthesia produced a large increase
in creatine excretion due to liver injury (143). Koven & Beard (45)
observed increases in creatine excretion after administering ether and
chloroform to the rat. Urethane increased creatine, but not creatinine
excretion, while amytal increased the excretion of both. Miller (144)
observed a pronounced creatinuria in the dog after anaphylactic
shock produced by the injection of horse serum. Masuda (145) stated
that the liver was unable to convert creatine into creatinine. Noboru
(146) showed that only a small part of creatine fed to rabbits is ex-
creted as creatine and creatinine. Parfentjev & Perlzweig (147) ob-
served that mouse urine contains 45 per cent of its total creatinine in
the form of creatine.

It is generally believed that the creatinine excretion in a given
individual fed on a creatine-free diet is constant (111). Variations in

the quantity of creatinine excreted have often been observed in the studies of Beard *et al.* Zwarenstein (90) also found marked variations in creatinine excretion. Hobson (105) noted that the daily excretion of creatinine by ninety-seven subjects varied from 1,430 to 3,800 mg.— a variation of 165 per cent. Hobson does not believe that the creatinine excretion of Folin's (111) subjects represents the normal creatinine output (cf. 148). Beard (46, 47) observed that the creatinine nitrogen excretion of four hundred normal medical students fed upon their usual diet varied from 0.7 to 1.9 gm. per day. It was concluded that creatinine excretion is not dependent upon the body weight of the individual (33). Hobson (105) stated that physical fitness also has no influence upon creatinine excretion.

Meat, aside from its small creatine content, is very important in the creatinuria that follows upon the administration of glycine to man. The degree of creatinuria observed in the case of HHB was always twice as great as in the case of PP (38). The same was true of the consumption of meat by these subjects. It is therefore possible that the negative results on creatine excretion by women, reported by Hyde (149) after glycine ingestion on meat-free diets, may be explained on this basis.

The relation of creatine and creatinine to water metabolism.—If it be true that creatinine can be hydrated to creatine in the body it is easy to visualize the role of water or saline in this connection. This view receives additional support from the finding of Rittenberg & Schoenheimer (55) that mice can synthesize alternate hydrogen atoms of cholesterol from the heavy water fed them. Beard *et al.* (38) studied the effect of injecting water and physiological saline into rats, and of their ingestion by man, upon muscle creatine formation by rats and upon creatine-creatinine excretion by the rat and man. It was observed that, in contrast to creatinine, water or saline did not increase muscle creatine. This indicated that the increased creatinuria observed under these conditions was not due to a liberation of muscle creatine. The injection of water and saline together caused exactly twice as much creatine excretion as the injection of either alone. In these studies creatine was still being excreted for two weeks or more after the injection of the extra water or saline. Of much interest in this connection is the observation of Hevesy & Hofer (150), who found that a molecule of heavy water remains in the body for about two weeks.

The ingestion of much extra water by subjects HHB and PP caused large increases in both creatine and creatinine excretion (38).

In several other studies with purines and hormones, the creatinuria was evidently due to the injection of water alone (32, 39, 41). It is also of interest in this connection that Orr & Rumold (151) showed that the administration of sodium chloride to dogs with pyloric obstruction led to greater increases in nonprotein nitrogen and creatinine in the blood than did the administration of water alone. Ide & Hongo (152) showed that the total creatinine of gastric juice rises and falls with its chloride content.

The feeding of sodium bicarbonate or disodium phosphate to rats greatly increased the transformation of creatinine to creatine (38). Keith & Osterberg (153) and Mason & Hellbaum (154) have shown that alkalosis increases the water content of tissues. Morgulis & Osheroff (155) using dystrophic rabbits observed a marked increase in sodium, chloride, and calcium, and Morgulis & Spencer (156) noticed a marked diuresis and increase in creatine and phosphate excretion. Fenn & Goettsch (157) also found considerable increases in extracellular water in nutritional muscular dystrophy. Hawk & Fowler (158) many years ago reported a total excretion of 1.5 gm. of creatine by their subject who drank 3 l. of water daily with his meals.

Browne et al. (159) showed that the nonadapted animal, like the animal exhausted by continuous exposure, responds to damage—such as may be induced by cold, excessive muscular exercise, or toxic doses of formaldehyde—with a decrease in water and chloride excretion followed by marked increases in creatine excretion. The adapted animal showed the reverse changes. White & Heinbecker (160) observed that after the onset of permanent polyuria in dogs suffering from diabetes insipidus, the creatinine clearance fell to about half its previous value. Creatine metabolism in diabetes insipidus has also been studied by Pakozdy (161), Caccuri (162), and D'Antona (163). Cutting et al. (164) showed that after massive infusions of physiological saline, the duodenum, muscle, spleen, and skin store water and saline in the proportion necessary to make a 1 per cent solution. From the above results it is possible that creatine can be formed from creatinine in situ in tissue hydremia.

There is a decreased rate of excretion of creatinine, uric acid, and chlorides after exercise (165). After strenuous muscular exercise there is no increase in the excretion of creatinine (166) but creatine excretion does occur (106). Since creatinine may be transformed into creatine in the presence of extra water in the tissues and since fatigued muscles take up additional water (167), it is logical to expect that the creatinuria of exercise may have its origin in part in the

creatinine of the tissues. It is also possible that some of the creatinuria of nutritional muscular dystrophy (157, 168, 169) and congestive heart failure (170) may be explained on this basis, since there is an increase in water in the former, and of interstitial fluids in the latter.

The transformation of creatinine to creatine in relation to water metabolism is also under the influence of some of the hormones. In six studies Beard *et al.* (34, 35, 39, 40, 41, 42) attributed the creatinuria after injection of several different hormones to water and salt retention.

In seven experiments the injection of pitressin caused a retention of 324 mg. of body creatinine in the rat, which was accompanied by an increased creatine excretion (expressed as creatinine) of 327 mg. (42). In four experiments injection of pitocin caused a body creatinine retention of 223 mg. which was accompanied by an increased creatine excretion of 254 mg. (42). In still other studies no creatinine was retained under these conditions but increases of from 22 to 150 mg. of urinary creatine were observed. It was concluded that these posterior pituitary hormones may cause a quantitative transformation of body creatinine into creatine on the one hand and stimulate creatine excretion on the other. These hormones do not, however, influence the metabolism of administered creatinine.

LITERATURE CITED

1. BOLLIGER, A., *J. Proc. Roy. Soc., N. S. Wales,* 70, 357 (1937)
2. BOLLIGER, A., *J. Proc. Roy. Soc., N. S. Wales,* 71, 40 (1937)
3. BOLLIGER, A., *J. Proc. Roy. Soc., N. S. Wales,* 71, 60 (1937)
4. GAEBLER, O. H., AND KELTCH, A. K., *J. Biol. Chem.,* 76, 357 (1928)
5. BOHN, H., AND HAHN, F., *Z. klin. Med.,* 125, 458 (1933)
6. MATSUMOTO, M., *Japan. J. Med. Sci., II, Biochem.,* 2, 205 (1933)
7. MILLER, B. F., AND DUBOS, R. J., *J. Biol. Chem.,* 121, 429 (1937)
8. MILLER, B. F., AND DUBOS, R. J., *J. Biol. Chem.,* 121, 447 (1937)
9. MILLER, B. F., AND DUBOS, R. J., *J. Biol. Chem.,* 121, 457 (1937)
10. MILLER, B. F., ALLINSON, M. J. C., AND BAKER, Z., *J. Biol. Chem.,* 130, 383 (1939)
11. BAKER, Z., AND MILLER, B. F., *J. Biol. Chem.,* 130, 393 (1939)
12. SULLIVAN, M. X., AND IRREVERRE, F., *J. Biol. Chem.,* 128, ix, ci (1939)
13. MILLER, B. F., AND BAKER, Z., *Rept. Proc. Third Intern. Congr. Microbiol.,* 215 (1940)
14. DUBOS, R., *Bact. Rev.,* 4, 1 (1940)
15. DUBOS, R., AND MILLER, B. F., *Proc. Soc. Exptl. Biol. Med.,* 39, 65 (1938)
16. GERARD, R. W., AND TUPIKOVA, N., *J. Cellular Comp. Physiol.,* 12, 325 (1938)
17. MYERS, V. C., AND MANGUN, G. H., *J. Biol. Chem.,* 132, 701 (1940)
18. MYERS, V. C., AND MANGUN, G. H., *J. Lab. Clin. Med.,* 26, 198 (1940)

19. BEARD, H. H., AND BARNES, B. O., *J. Biol. Chem.*, **94**, 49 (1931)
20. BEARD, H. H., AND BOGGESS, T. S., *Am. J. Physiol.*, **113**, 647 (1935)
21. TRIPOLI, C. J., AND BEARD, H. H., *Arch. Internal Med.*, **53**, 435 (1934)
22. TRIPOLI, C. J., MCCORD, W. M., AND BEARD, H. H., *J. Am. Med. Assoc.*, **103**, 1595 (1934)
23. BEARD, H. H., TRIPOLI, C. J., AND ANDES, J. E., *Am. J. Med. Sci.*, **188**, 706 (1934)
24. TRIPOLI, C. J., AND BEARD, H. H., *Southern Med. J.*, **31**, 662 (1938)
25. BEARD, H. H., *Am. J. Physiol.*, **124**, 530 (1938)
26. BOGGESS, T. S., AND BEARD, H. H., *J. Biochem. (Japan)*, **27**, 231 (1938)
27. BEARD, H. H., AND PIZZOLATO, P., *J. Biochem. (Japan)*, **28**, 421 (1938)
28. BEARD, H. H., ESPENAN, J. K., AND PIZZOLATO, P., *Am. J. Physiol.*, **127**, 716 (1939)
29. BEARD, H. H., *J. Biol. Chem.*, **133**, vii (1940)
30. KELLY, C. J., AND BEARD, H. H., *J. Biochem. (Japan)*, **29**, 155 (1938)
31. BEARD, H. H., AND PIZZOLATO, P., *J. Pharmacol.*, **63**, 306 (1938)
32. KOVEN, A. L., AND BEARD, H. H., *J. Pharmacol.*, **68**, 80 (1940)
33. BEARD, H. H., AND JACOB, E. J., *Arch. Neurol. Psychiat.*, **42**, 67 (1939)
34. PIZZOLATO, P., AND BEARD, H. H., *Endocrinology*, **24**, 358 (1939)
35. KOVEN, A. L., AND BEARD, H. H., *Endocrinology*, **25**, 221 (1939)
36. BRAZDA, F. G., ESPENAN, J. K., AND BEARD, H. H., *Pacific Coast Med.*, **7**, 38 (1940)
37. BEARD, H. H., AND ESPENAN, J. K., *Enzymologia*, **8**, 241 (1940)
38. BEARD, H. H., ESPENAN, J. K., KOVEN, A. L., AND PIZZOLATO, P., *J. Biol. Chem.*, **133**, x (1940)
39. BEARD, H. H., AND JACOB, E. J., *Endocrinoolgy*, **26**, 1064 (1940)
40. BEARD, H. H., AND JACOB, E. J., *Endocrinology*, **26**, 1069 (1940)
41. KOVEN, A. L., PIZZOLATO, P., AND BEARD, H. H., *Endocrinology*, **27**, 95 (1940)
42. BEARD, H. H., AND PIZZOLATO, P., *Endocrinology*, **27**, 908 (1940)
43. BEARD, H. H., AND ESPENAN, J. K., *Am. J. Physiol.* (In press)
44. BEARD, H. H., AND BOGGESS, T. S., *J. Biol. Chem.*, **114**, 771 (1936)
45. KOVEN, A. L., AND BEARD, H. H., *Pacific Coast Med.*, **6**, 34 (1939)
46. BEARD, H. H., *Human Biol.*, **4**, 351 (1932)
47. BEARD, H. H., *Human Biol.*, **7**, 419 (1935)
48. BEARD, H. H. (In press)
49. BLOCH, K., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **133**, 633 (1940)
50. BLOCH, K., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **134**, 785 (1940)
51. RATNER, S., SCHOENHEIMER, R., AND RITTENBERG, D., *J. Biol. Chem.*, **134**, 653 (1940)
52. FOSTER, G. L., SCHOENHEIMER, R., AND RITTENBERG, D., *J. Biol. Chem.*, **127**, 319 (1939)
53. RITTENBERG, D., SCHOENHEIMER, R., AND KESTON, A. S., *J. Biol. Chem.*, **128**, 603 (1939)
54. SCHOENHEIMER, R., RATNER, S., AND RITTENBERG, D., *J. Biol. Chem.*, **130**, 703 (1939)
55. SCHOENHEIMER, R., AND RITTENBERG, D., *Physiol. Rev.*, **20**, 218 (1940)
56. BLOCH, K., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **135**, 99 (1940)
57. ABBOTT, L. D., AND LEWIS, H. B., *J. Biol. Chem.*, **131**, 479 (1939)

58. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.,* **131,** 111 (1939)
59. Braunstein, A. E., and Kritsmann, M. G., *Biokhimiya,* **2,** 242 (1937)
60. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.,* **132,** 559 (1940)
61. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.,* **134,** 627 (1940)
62. Vigneaud, V. du, Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.,* **131,** 57 (1939)
63. Vigneaud, V. du, Chandler, J. P., Cohn, M., and Brown, G. W., *J. Biol. Chem.,* **134,** 787 (1940)
64. Thomas, K., *J. Nutrition,* **2,** 419 (1930)
65. Rose, W. C., *Ann. Rev. Biochem.,* **2,** 187 (1933)
66. Fisher, R. B., and Wilhelmi, A. E., *J. Biol. Chem.,* **132,** 135 (1940)
67. Baker, Z., and Miller, B. F., *J. Biol. Chem.,* **132,** 233 (1940)
68. Shibuya, S.-I., *J. Biochem. (Japan),* **25,** 701 (1937)
69. Shibuya, S.-I., *J. Biochem. (Japan),* **29,** 339 (1939)
70. Sasaki, T., *J. Chosen Med. Assoc.,* **20,** 1821 (1930)
71. Bach, S. J., *Biochem. J.,* **33,** 90 (1939)
72. Fisher, R. B., and Wilhelmi, A. E., *Biochem. J.,* **31,** 1136 (1937)
73. Davenport, H. W., Fisher, R. B., and Wilhelmi, A. E., *Biochem. J.,* **32,** 262 (1938)
74. Fitzgerald, J. G., and Schmidt, C. L. A., *Proc. Soc. Exptl. Biol. Med.,* **10,** 55 (1912)
75. Sears, H. J., *J. Infectious Diseases,* **19,** 105 (1916)
76. Sears, H. J., *J. Bact.,* **2,** 187 (1917)
77. Fish, C. H., and Beckwith, T. D., *J. Bact.,* **37,** 111 (1939)
78. Stekol, J. A., and Schmidt, C. L. A., *Univ. Calif. Pub. Physiol.,* **8,** 31 (1933)
79. Griffith, W. H., and Mulford, D. J., *Proc. Soc. Exptl. Biol. Med.,* **45,** 657 (1940)
80. Hoppe-Seyler, F., *Z. Biol.,* **20,** 433 (1930)
81. Challenger, F., *J. Soc. Chem. Ind.,* **55,** 900 (1936)
82. Fosse, R., and Larambergue, R. de, *Compt. rend.,* **204,** 1285 (1937)
83. Fosse, R., and Larambergue, R. de, *Compt. rend.,* **205,** 188 (1937)
84. Mourot, G., *Bull. soc. chim. biol.,* **19,** 1209 (1937)
85. Milhorat, A. T., and Toscani, V., *J. Biol. Chem.,* **114,** 461 (1936)
86. Abderhalden, E., and Buadze, S., *Z. ges. exptl. Med.,* **65,** 1 (1929)
87. Abderhalden, E., and Buadze, S., *Z. ges. exptl. Med.,* **66,** 635 (1929)
88. Abderhalden, E., and Buadze, S., *Z. ges. exptl. Med.,* **69,** 651 (1930)
89. Chrometzka, F., *Z. ges. exptl. Med.,* **86,** 483 (1933)
90. Zwarenstein, H., *Biochem. J.,* **20,** 742 (1926)
91. Vague, J., and Dunan, J., *La Creatine. Etude Physio-pathologique* (Masson et Cie, Paris, 1940)
92. Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice,* 2nd ed., p. 990 (Williams & Wilkins Co., Baltimore, 1940)
93. Hastings, A. B., Kistiakowsky, G. B., Cramer, R. D., Klemperer, F. W., Solomon, A. K., and Vennesland, B., *Science,* **91,** 421 (1940)
94. Sacks, J., *Am. J. Physiol.,* **122,** 215 (1938)
95. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.,* **81,** 629 (1929)
96. Perlman, I., Stillman, N., and Chaikoff, I. L., *J. Biol. Chem.,* **135,** 355 (1940)

97. ALMQUIST, H., AND MECCHI, E., *J. Biol. Chem.,* **135,** 355 (1940)
98. SHAPIRO, B. G., *J. Physiol.,* **92,** 178 (1938)
99. SCHOO, A. G., AND BOER, J., *Nederland. Tijdschr. Geneeskunde,* **78,** 1 (1934)
100. CAMERON, J. D. S., *Quart. J. Exptl. Physiol.,* **23,** 351 (1933)
101. ESPERSEN, T., AND THOMSEN, A., *Hospitalstidende,* **80,** 85 (1937)
102. ISHIKAWA, M., *Osaka Ig. Kw. Z.,* **31,** 2059 (1932)
103. LINEGAR, C. R., FROST, T. T., AND MYERS, V. C., *Arch. Internal Med.,* **61,** 430 (1938)
104. DJEN, L. D., AND PLATT, B. S., *Trans. Congr. Far East. Assoc. Trop. Med., 9th Congr.,* **I,** 379 (1934)
105. HOBSON, W., *Biochem. J.,* **33,** 1425 (1939)
106. HALDI, J., AND BACHMANN, G., *Am. J. Physiol.,* **115,** 364 (1936)
107. TAYLOR, F. H. L., AND CHEW, W. B., *Am. J. Med. Sci.,* **191,** 256 (1936)
108. WANG, E., *Acta Med. Scand., Suppl.,* 105 (1939)
109. THOMASEN, A., *Thesis* (Inst. Med. Physiol., Copenhagen, 1938)
110. WERNER, E. A., *The Chemistry of Urea* (Longmans Green & Co., New York, 1923)
111. FOLIN, O., *Am. J. Physiol.,* **13,** 117 (1905)
112. YUDELES, A. L., AND SHRETTER, A. V., *Arch. sci. biol. U.S.S.R.,* **40,** 65 (1936)
113. PASCHKIS, K., AND SCHWONER, A., *Arch. intern. pharmacodynamie,* **52,** 225 (1936)
114. DEGAN, C., *Bull. soc. chim. biol.,* **19,** 686 (1937)
115. MILHORAT, A. T., *Deut. Arch. klin. Med.,* **174,** 487 (1933)
116. GRADINESCU, A., AND DEGAN, C., *Compt. rend.,* **124,** 79 (1937)
117. DEGAN, C., *Bull. soc. chim. biol.,* **19,** 1325 (1937)
118. DEGAN, C., *Compt. rend.,* **127,** 451 (1938)
119. NOGAMI, K., *Osaka Ig. Kw. Z.,* **28,** 1483 (1929)
120. GONZALEZ-RUBIERA, J. A., *Rev. Univ. Habana,* No. 4, 59 (1930)
121. SEKINE, M., *Osaka Ig. Kw. Z.,* **27,** 349 (1928)
122. DEGAN, C., *Bull. soc. chim. biol.,* **20,** 373 (1938)
123. FELIX, K., AND MÜLLER, H., *Z. physiol. Chem.,* **240,** 1 (1936)
124. BACHMANN, G., HALDI, J., ENSOR, C., AND WYNN, W., *Am. J. Physiol.,* **124,** 77 (1938)
125. KYOGOHU, K., *Arb. med. Fakultät Okayama,* **5,** 503 (1938)
126. SUGIYAMA, G., *J. Biochem. (Japan),* **29,** 97 (1939)
127. MILHORAT, A. T., HARDY, J. D., BARTELS, W. E., AND TOSCANI, V., *Proc. Soc. Exptl. Biol. Med.,* **45,** 397 (1940)
128. MIYAZAKIM, K., *Sei-i-kai Med. J.,* **56,** 2085 (1937)
129. TERROINE, E. F., BERNARDIE, A. M. DE LA, AND LELU, P., *Compt. rend.,* **204,** 1757 (1937)
130. CUTHBERTSON, D. P., MCGIRR, J. L., AND ROBERTSON, J. S. M., *Quart. J. Exptl. Physiol.,* **29,** 13 (1939)
131. MORI, G., *Sei-i-kai Med. J.,* **54,** 1805 (1936)
132. TERROINE, E. F., AND NATAF, B., *Ann. physiol. physicochim. biol.,* **14,** 145 (1938)
133. KAZIRO, K., AND TAKU, A., *J. Biochem. (Japan),* **11,** 203 (1929)

134. Taku, A., *J. Biochem. (Japan)*, **12**, 371 (1930)
135. Ikoma, S., *Mitt. med. Ges. Okayama*, **40**, 890 (1928)
136. Masai, Y., and Fukutomi, T., *Osaka Ig. Kw. Z.*, **25**, 933 (1926)
137. Terroine, E. F., Gaija, A., and Bayle, L., *Bull. soc. chim. biol.*, **14**, 900 (1932)
138. Pariset, G., *Compt. rend.*, **197**, 704 (1933)
139. Terroine, E. F., and Boy, G., *Compt. rend.*, **197**, 702 (1933)
140. Terroine, E. F., *Biochem. Z.*, **293**, 435 (1937)
141. Zamagi, V., *Atti accad. Lincei, Classe sci. fis. mat. nat.*, **23**, 629 (1936)
142. Daft, F. S., Robscheit-Robbins, F. S., and Whipple, G. H., *J. Biol. Chem.*, **121**, 45 (1937)
143. Daft, F. S., Robscheit-Robbins, F. S., and Whipple, G. H., *J. Biol. Chem.*, **113**, 391 (1936)
144. Miller, L. L., *J. Biol. Chem.*, **133**, 93 (1940)
145. Masuda, K., *J. Chosen Med. Assoc.*, **19**, 538 (1929)
146. Noboru, S., *Sei-i-kwai Med. J.*, **47**, 1 (1928)
147. Parfentjev, I. A., and Perlzweig, W. A., *J. Biol. Chem.*, **100**, 551 (1933)
148. Jellinek, E. M., and Looney, J. M., *J. Biol. Chem.*, **128**, 621 (1939)
149. Hyde, E., *J. Biol. Chem.*, **134**, 95 (1940)
150. Hevesy, G., and Hofer, E., *Klin. Wochschr.*, **13**, 1524 (1934)
151. Orr, T. G., and Rumold, M. J., *Arch. Surg.*, **37**, 295 (1938)
152. Ide, H., and Hongo, Y., *Nagasaki Ig. Kw. Z.*, **10**, 865 (1932)
153. Keith, N. M., and Osterberg, A. E., *Am. J. Physiol.*, **123**, P114 (1938)
154. Mason, E. C., and Hellbaum, A. A., *Am. J. Physiol.*, **123**, 143 (1938)
155. Morgulis, S., and Osheroff, W., *J. Biol. Chem.*, **124**, 767 (1938)
156. Morgulis, S., and Spencer, H. C., *J. Nutrition*, **12**, 191 (1936)
157. Fenn, W. O., and Goettsch, M., *J. Biol. Chem.*, **120**, 41 (1937)
158. Hawk, P. B., and Fowler, C. C., *J. Exptl. Med.*, **12**, 388 (1910)
159. Browne, J. S. L., Karády, S., and Selye, H., *J. Physiol.*, **97**, 1 (1939)
160. White, H. L., and Heinbecker, P., *Am. J. Physiol.*, **123**, 566 (1938)
161. Pakozdy, K., *Orvosi Hetilap*, **73**, 1167 (1929)
162. Caccuri, S., *Arch. pat. clin. med.*, **14**, 266 (1934)
163. D'Antona, L., *Arch. ist. biochim. ital.*, **3**, 187 (1931)
164. Cutting, R. A., Lands, A. M., and Larson, P. S., *Arch. Surg.*, **36**, 586 (1938)
165. Norris, E. R., and Weiser, R. S., *Am. J. Physiol.*, **119**, 642 (1937)
166. Krüger, F. v., *Arbeitsphysiol.*, **10**, 8 (1938)
167. Shafer, G. D., and Skow, R. K., *Am. J. Physiol.*, **122**, 551 (1938)
168. Goettsch, M., and Brown, E. P., *J. Biol. Chem.*, **97**, 549 (1932)
169. Knowlton, G. C., Hines, H. M., and Brinkhous, K. M., *Proc. Soc. Exptl. Biol. Med.*, **41**, 453 (1939)
170. Hermann, G., *Intern. Clinics*, **3**, 139 (1937)
171. Hunter, A., *Creatine and Creatinine* (Longmans Green & Co., New York, 1928)

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DETOXICATION MECHANISMS

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This review comprises a limited discussion of selected topics which appeared in the literature from 1937 to October, 1940. The headings of the topics covered indicate the reaction or the substance participating in what traditionally is considered a detoxication reaction. The term, detoxication, calls for some elucidation. It implies either total abolition or reduction in toxicity of a substance as a result of certain changes which it undergoes in the animal body. The detoxication product therefore should be either harmless or less toxic than the parent substance. Some of the reactions reviewed here comply with this definition. In some instances, however, nothing is known about the relative toxicity of the parent substance and the detoxication product; in others, the detoxication product is apparently more toxic than the parent substance. In some cases, rendering the substance harmless *in vivo* induces disturbances in the animal which lead to a cessation of growth and injury to vital organs, while in others, a detoxication product may be as harmless as the original substance. Clearly then, the so-called detoxication mechanisms as presented here under several well-known biochemical reactions, should be regarded, not as special mechanisms provoked to action in the animal body upon introduction of a foreign substance into the organism, but merely as a portion of what might be termed the metabolism of organic compounds. Nor do we wish to give the impression that this is a review of the fate of "foreign substances" in the animal body. Some of the so-called foreign substances or the reactions which were thought to be special mechanisms for the disposal of the foreign compounds have proved in recent years to be of vital importance to the animal's well-being, and the reactions which lead to their disposal from the animal body have been found to be commonplace.

Oxidation, reduction, hydrolysis.—Bernhard reported numerous experiments on the fate of hydrogenated carbocyclic compounds in the animal body. Thus, (1) the administration of $C_6H_{11}COOH$ to dogs yielded benzoic acid and $C_6H_{11}CONHCOOH$ in the urine. The latter substance was postulated to be an intermediate in the conversion of hydrogenated benzoic acid to benzoic acid. Monomethyl or dimethyl-

N-hydrogenated benzamide also yielded benzoic acid. *Cis-* or *trans-o*-methyl-hydrogenated benzoic acid and l-1,2,4,5(OH)$_4$C$_6$H$_7$COOH were not dehydrogenated but were excreted in the urine unchanged. Hydrogenated phenol, toluol, *o-* or *p*-dimethylbenzene, or hydrogenated aniline are oxidized in the dog. After hydrogenated phenylacetic acid was fed, no phenylacetic or phenaceturic acid could be detected in the urine, although some succinic acid could be isolated. m-CH$_3$ · C$_6$H$_{10}$COOH was dehydrogenated to a large extent, but the corresponding para isomer only partially. The three isomeric methyl benzoic acids were excreted by the dog unconjugated. In further confirmation of the beta oxidation theory of fatty acids, it was found (2) that adipylmonoanilide and sebacylmonoanilide, when fed to dogs, yielded in the urine succinanil and succinyl-monoanilide.

Torboli (3) found cyclohexane and cyclohexene equally toxic to rabbits on intraperitoneal injection. A compound, probably adipic acid, was isolated from the urine of rabbits which were injected with either of the substances. Bernhard (4), on the other hand, reported that cyclohexane disappeared completely when it was fed or injected into rabbits or dogs. Cyclohexyl derivatives of butyric, valeric, and decanoic acids were fed to dogs and only those acids which by beta oxidation could yield benzoic acid were excreted in the urine, while those acids which could form phenylacetic acid disappeared. Cyclohexylaminobutyric acid yielded benzoic acid in dog urine, while cyclohexylaminopropionic acid was largely burned, but both compounds were excreted in part as optically active N-acetyl derivatives. The three isomers of hexahydrophthalic acid were excreted unchanged by the dog, while the three isomers of hexahydroaminobenzoic acid were completely utilized (5). Administration of the *o-* and especially the *p*-hexahydroaminobenzoic acids increased the excretion of oxalic acid in the urine of dogs. *Cis-trans*-decahydroquinoline or *cis-trans*-decahydroisoquinoline were also completely utilized when these substances were fed to dogs in small daily doses. 1,2,3,4-Tetrahydroquinoline yielded 25 per cent of the theoretical amount of 2-hydroxyquinoline, while 1,2,3,4-tetrahydroisoquinoline was oxidized (6). It was further shown (7) that in dogs decahydronaphthalene was partially destroyed, but a portion was excreted as decahydronaphthol. Tetrahydronaphthalene behaved similarly. The larger part of decahydro-β-naphthol was oxidized when it was fed to dogs, but about 30 per cent was excreted unchanged together with some decahydronaphthodiol. Furthermore, it was found that the carboxyl group in the beta position facili-

tated the oxidation of naphthoic acids in the dog. No compound mentioned above was conjugated with glucuronic acid in the dog.

If the disappearance of the substance is taken to indicate its complete destruction *in vivo*, certain generalizations may be made from Bernhard's work. Hydrogenation of an aromatic compound facilitates its complete destruction *in vivo*. The dog is able to dehydrogenate certain substances, and the degree of dehydrogenation may be influenced by the nature and position of substituents in the completely or partially hydrogenated ring.

Drummond & Finar (8), following up the work of Jaffé (9), reported that benzene, when fed to rabbits, yielded *trans-trans*-muconic acid in the urine, although *cic-cis*-muconic acid could be epected from the opening of the benzene ring. These workers found that *trans-trans*-muconic acid is readily formed from *cis-cis*-muconic acid when the latter is added to the urine of rabbits which received benzene. Normal rabbit urine did not bring about the transformation. Confirming reports of others (10), Drummond & Finar believe that phenylmercapturic acid is also formed from benzene in rabbits.

Jorissen (11) reported the oxidation of naphthalene by ascorbic acid *in vitro* to yield substances which responded to fluorescein reaction (phthalic acid, naphthoquinone, naphthohydroquinone, etc.). These experiments are of interest in connection with the observed decrease in ascorbic acid content of the aqueous humor and lens in animals poisoned by naphthalene (12). Mueller (13) reported that naphthalene-poisoned animals exhibit deranged carbohydrate metabolism, as is indicated by a decreased resorption of glucose by the lens of the animal, and an increase in the lactic acid content of the blood, aqueous humor, and lens.

Bernheim *et al.* (14) studied the action of *p*-aminophenol on certain tissue oxidations. These workers (15) also found that acetanilide is hydrolyzed rapidly by the rat liver and kidney tissue, more slowly by other tissues, and not at all by heart and skeletal muscle. Evidence was presented which indicated that a part of the aniline liberated was oxidized to *p*-aminophenol by various tissues. Marshall & Walzl (16) suggested that the cyanosis which is often observed after sulfanilamide therapy was due to some black oxidation product of the drug. Meulengracht & Lundsteen (17) attribute cyanosis and anemia in chronic acetanilide poisoning to *p*-aminophenol and its derivatives and not to methemoglobin. Wendel *et al.* (18) find methemoglobin as the principal abnormal pigment in the blood of subjects showing cyanosis from

sulfanilamide. Scudi (19) isolated monohydroxyl sulfapyridine and its glucuronic acid derivatives from the urine of animals which were fed sulfapyridine. Shelswell & Williams (20) reported data to show that sulfanilamide, when fed to rabbits, is excreted in the urine as a hydroxy derivative conjugated with sulfate, to the extent of 6 to 12 per cent of the dose fed. James (21) isolated p-N-acetylhydroxyl-aminobenzenesulfonamide, p-hydroxylaminobenzenesulfonic acid, and p-aminophenol from the urine of patients receiving sulfanilamide. The isolated substances appeared to have been excreted as sulfates and glucuronates. p-Aminophenol underwent further change with the formation of a pigment which was excreted in the urine. James attributes some of the toxic effects of sulfanilamide to hydroxylamine derivatives and to p-aminophenol. He found (22) that oxidation of sulfanilamide and sulfapyridine by hydrogen peroxide yields, among other products, hydroxylamine derivatives and p-aminophenol. Rosenthal & Bauer (23) have also demonstrated the presence of aromatic hydroxylamines in the urine of subjects fed sulfanilamide. They suggest that the effectiveness of sulfanilamide may be due to its action on catalase. Shinn et al. (24) reported the conversion of sulfanilamide to a p-hydroxylamine derivative by the action of ultraviolet irradiation on the drug. Mann & Keilin (25) found sulfanilamide to be a specific inhibitor of carbonic anhydrase. Other enzymes, including catalase, were unaffected by sulfanilamide. The literature on the pharmacology and therapy of sulfanilamide and related compounds has been reviewed on several occasions (26 to 31).

Boyland & Levi (32) and later Dobriner, Rhoads & Lavin (33) administered 1,2,5,6-dibenzanthracene to rabbits, rats, and mice and, from the feces and urine, isolated dihydroxy derivatives of the carcinogen. The hydroxyl groups of the isolated substances are, apparently, at some positions other than 4,8,9, or 10 (32). The isolated dihydroxy derivatives were not the same for the three species (33). Chalmers (34) injected a colloidal solution of 3,4-benzpyrene into rats and isolated from the excreta a photolabile, alkali-soluble product with green-yellow fluorescence whose nature has not yet been determined. He does not rule out the possibility of the formation of a mercapturic acid from 3,4-benzpyrene in the rat. Neufach (35) reviewed the literature on the chemical changes undergone by 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, methylcholanthrene, and anthracene in vivo.

Stroud (36) isolated 4-methoxy-4′-hydroxydiphenyl ether from the urine of rabbits injected with 4,4′-methoxydiphenyl ether. 4-Me-

thoxybiphenyl yielded in the urine 4,4′ dihydroxybiphenyl. He offers these data as evidence for demethylation *in vivo* to support the suggestion (37) that estrone methyl ether is metabolized to estrone. He further found (38) that in the rabbit, over 25 per cent of administered diphenyl could be isolated from the urine as 4-hydroxydiphenyl; over 22 per cent of diphenyl ether, as hydroxydiphenyl ether; and 15 per cent of diphenyl methane, as 4-hydroxydiphenyl methane. Three per cent of administered stilbene was isolated as 4,4′-dihydroxystilbene and 34 per cent as benzoic acid. The administration of γ-δ-diphenyl-β-δ-hexadiene to rabbits yielded in the urine a highly active estrogenic phenol, probably 4,4′-dihydroxy-γ-δ-diphenyl-β-δ-hexadiene.

CONJUGATION REACTIONS

Glucuronic acid.—Goebel (39) synthesized 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester and compared it to the product formed by the acetylation of the methyl ester of the benzoic acid-glucuronic acid compound which was isolated from the urine of dogs which were fed sodium benzoate. The compounds were identical. The claim of Pryde & Williams (40) that the glucuronic acid conjugate with benzoic acid is benzoylglucuronate and not glucuronic acid monobenzoate (41) is thus established.

Several compounds can be added to the list of those known to conjugate with glucuronic acid *in vivo*. Di Somma (42) isolated phenolphthalein-monoglucuronide from the urine of rabbits and guinea pigs injected with phenolphthalein. Trichloroethanol, like tribromoethanol, is excreted in the urine of rabbits in part as the glucuronate (43). The corresponding glucuronides were isolated from the urine of rabbits and humans upon administration of *d*-isomenthol (44), *d*-neomenthol (45), cinnamic acid (46), and sulfapyridine (19). Administration of *l*-menthone to rabbits yielded *d*-neomenthylglucuronide in the urine, indicating partial reduction of the carbonyl group (45). *d*-Isomenthone was also reduced in the rabbit and excreted as *d*-isomenthylglucuronide (45). Kuhn & Low (47) isolated menthoglycol glucuronide from the urine of rabbits after the administration of citronellal. The authors proposed that citronellal was converted to *p*-menthan-3,8-diol in the stomach before the conjugation with glucuronic acid occurred elsewhere. Schmid (48) found that in the frog, menthol conjugates with glucuronic acid in the liver. Bliss & Glass (49) observed no difference in toxicity of the eight isomers of menthol in rabbits, rats, and humans.

administered drugs *in vivo,* but it was proposed that the increased synthesis of the vitamin by the rat is related to the detoxication of the administered drugs *in vivo,* and that the endogenous production of ascorbic acid originates from tissue metabolites in much the same way as that which Lipschitz & Bueding (68) demonstrated in the case of glucuronic acid.

Glycoside formation in plants.—It has been recognized for some time that glycoside formation in plants may serve to render less active and to stabilize reactive substances arising in the course of plant metabolism (80). It has now been demonstrated that plants can also form glycosides from substances which do not occur normally in plants. Thus, exposure of corms of the Alice Tiplady variety or potatoes to ethylene chlorohydrin led to the synthesis by the plants of β-(2-chloroethyl)-*d*-glucoside which was isolated and identified (81). Exposure of gladiolus corms and tomato plants to *o*-chlorophenol, chloral hydrate, or trichloroethanol led to the formation of the corresponding gentiobiosides in the plant (82). These glycosides do not move readily in the plant, for when the tubers which contained them were planted the resulting sprouts were free of the glycosides. Miller (82) offers these data as evidence "that detoxication may be one role of glycoside formation in plants, if it is true, which seems likely, that the resulting glycosides are less harmful to the plant than the more reactive aglucons." These experiments are particularly interesting when it is recalled that the substances mentioned above form in the animal body the corresponding glucuronides.

Glycine.—Borsook & Dubnoff (83) were unable to detect benzamide or hippuric acid formation by the action of glycerol extracts of kidney or liver on benzoic acid, contrary to claims of Waelsch & Busztin (84). It was further shown that in the guinea pig, rabbit, and rat, the conjugation of benzoic acid with glycine occurs in the liver and kidney. In the dog, the synthesis takes place only in the kidney. Quick's observation (85) that benzoylglucuronate, when injected into dogs, yields hippuric acid, or that hippuric acid, when injected, yields benzoylglucuronate, suggested to Borsook & Dubnoff (83) the inference that from thermodynamic considerations the synthesis of hippuric acid in the dog or other animal is not necessarily a reversible reaction which obeys the law of mass action. Quick's observation may be an indication of hippuric acid synthesis in the kidney and its hydrolysis in the liver or other organ.

Schoenheimer *et al.* (86) using isotopic hydrogen and nitrogen as

metabolic labels reported that almost 75 per cent of the hippuric acid administered to rats was excreted in the urine without undergoing hydrolysis *in vivo*. Their results further indicated that benzoic acid conjugates *in vivo* directly with dietary glycine. Rittenberg & Schoenheimer (87), on the other hand, find that only one third of glycine containing isotopic nitrogen fed to rats with benzoic acid was excreted in the urine as hippuric acid. The greater portion of hippuric acid glycine originated from endogenous sources in spite of the abundant supply of dietary glycine. Waelsch & Rittenberg (88) find that 55 per cent of the glycine in hippuric acid which was isolated from the urine of rats which received benzoic acid and isotopic glycine was derived from the administered glycine. Whether these results can be harmonized by assuming that the labile, semilabile, or stable nature of the isotopes used influenced the quantitative aspect of the data, the reviewer is uncertain. Although it has not been proposed, it was denied that "mysterious labilizing enzymes" exist (89).

Terroine & Boy (90) reported that as much as 70 per cent of endogenous nitrogen loss can be converted to glycine for the synthesis of hippuric acid at the expense of urea. Administration of mineral acid together with benzoic acid did not influence the synthesis of glycine for hippuric acid formation.

Vasiliu *et al.* (91) reported several papers which attempt to show that the acid or alkaline nature of the diet influences the amount of hippuric or phenaceturic acid in the urine of mammals. It will be recalled that Griffith (92) showed that hippuric acid synthesis is unrelated to the basic or acidic nature of the diet.

Abbott & Lewis (93) found that sarcosine, but not N,N-dimethyl glycine and betaine, increased the rate of hippuric acid excretion in the rabbit. Inasmuch as N-N-dimethyl glycine and betaine apparently could not yield glycine, the authors suggested that oxidative deamination rather than hydrolysis of the N-methyl group of sarcosine occurred. Bloch & Schoenheimer (94), in analogous experiments in which the nitrogen of the amino group of sarcosine was replaced by isotopic nitrogen as a tracer, also observed the formation of hippuric acid from the administered benzoic acid and sarcosine. Their data showed, however, that hydrolysis of sarcosine to glycine occurred, since the isotopic nitrogen was found intact in the newly formed hippuric acid.

Waelsch & Rittenberg (88) came to the conclusion that glutathione does not exclusively furnish glycine for hippuric acid formation *in vivo*, contrary to the attractive hypothesis originally proposed by

Waelsch *et al.* (95), and commented upon by others (96), that glutathione may be the source of glycine, glutamic acid, and cysteine for detoxication purposes *in vivo*.

Quick (97) reported an improved procedure for clinical application of the hippuric acid liver function test. It is possible to mention only the bibliography of but a few applications of this test to various pathological conditions (98 to 107).

Sulfuric acid.—Williams (108) studied the influence of the dose and of ortho, meta, and para substitution of phenol on the sulfate detoxication of the drug in the rabbit. In the ortho position, acidic groups depress the conjugation by 50 per cent, while basic groups increase it by 50 per cent; neutral groups have no effect. The carboxyl group in ortho position abolishes the conjugation entirely. In the meta position, the influence is similar to that in the ortho position, but in the para position, groups of varying nature have little effect, with the exception of the carboxyl and amide groups. The latter group appears to increase the extent of conjugation with sulfuric acid, and the former to decrease it. The extent of phenol sulfate formation in the rabbit for doses lower than 100 mg. per kg. increases with decreasing doses, and no substance tested was found to yield the sulfate derivative to greater extent than 40 per cent. Conjugation of phenol with sulfuric acid occurs rapidly in the eviscerated, nephrectomized, and hepatectomized animals (109, 110, 111).

Blaschko *et al.* (112) find that many substances related to epinephrine are attacked by epinephrine oxidase of the guinea pig or rat liver primarily at the side chain of the molecule. The $\equiv C - CH_2 - N =$ grouping appear to be the essential configuration for the oxidation to occur. Richter (113) reported that the amines of the $RR' = CH - NH_2$ series such as ephedrine, benzedrine, etc., are excreted unchanged by man. He (114) later reported that corbasil, epinine, and *d-* or *l*-epinephrine, when fed by mouth to man in doses of 0.13 to 0.66 mg. per kilo, are excreted in the urine mainly in the conjugated form, probably as sulfate esters. Up to 70 per cent of the administered epinephrine could be accounted for in the urine after acid hydrolysis. The conjugation with sulfuric acid is believed to take place with one of the phenolic groups of epinephrine. No evidence for the excretion of epinine and epinephrine in the urine as protocatechuic acid was found. Loeper & Parrod (115) reported the synthesis of tyramine sulfuric acid, and Loeper *et al.* (116) found that tyramine sulfuric acid had no physiological action on rabbits on injection. Hydrolysis of the ester restored

the activity of tyramine. Whether tyramine conjugates *in vivo* with sulfuric acid remains to be demonstrated, although *a priori* such a reaction appears probable.

Wiley (117) administered β-naphthylamine to dogs and isolated from the urine 2-amino-1-naphtholsulfate, which was identified and synthesized. Tumors were produced in the bladder of animals by the administration of β-naphthlyamine (118).

Cleary *et al.* (119) observed a rise in the excretion of ethereal sulfates in the urine of dogs fed chlorinated naphthalenes. The neutral sulfur excretion remained unaffected. Evidence was presented for what the authors termed "metabolic dehalogenation."

Collier (120) reported the excretion of leucophenothiazone potassium sulfate in the urine of sheep fed phenothiazine. The latter substance was shown to be an effective anthelmintic for sheep (121).

Acetic acid.—Klein & Harris (122) find that the acetylation of sulfanilamide by rabbit and human liver slices is determined by the rate of acetate production by the tissues. Acetate as well as substances which give rise to acetate in the tissues increase the degree of acetylation of sulfanilamide. Under anaerobic conditions or in the presence of arsenious oxide, or iodoacetamide, a decrease in acetylation was observed which in the absence of added acetate was due mainly to an inhibition of acetate production. Stewart *et al.* (123) found that removal of liver from the rabbit checks the acetylation of sulfanilamide, while the removal of stomach, intestine, pancreas, spleen, or kidney does not. The authors conclude that liver is the site of acetylation of sulfanilamide. James (124) confirmed the observation of Klein & Harris (122) in that he also found an increased excretion of acetylsulfanilamide in the urine of mice when sodium acetate was fed along with sulfanilamide. Although the administration of acetate was definitely protective to mice against toxic effects of sulfanilamide, yet James confirms Marshall *et al.* (125) in the observation that acetylsulfanilamide is more toxic than sulfanilamide. Obviously, the acetylation of sulfanilamide can hardly be called a detoxication reaction. He suggests (124) that the toxicity of sulfanilamide may in part be due to *p*-aminophenol and *p*-hydroxyaminobenzenesulfonamide which were found in the urine, and in part to a sudden withdrawal from the body of the acetyl precursors, while "the administration of acetylsulfanilamide as such would not make this sudden demand but may well be injurious in other ways." Kohl & Flynn (126) find that the ease of the deacylation of acetyl, butyryl, valeryl, caproyl, and heptanoyl

derivatives of sulfanilamide and sulfanilylhydroxamide by the liver pulp increases with the length of the acyl group in the series.

DuVigneaud & Irish (127) revived the theory of Knoop (128) on the role of acetylation in the synthesis of amino acids *in vivo*. The theory proposes the interaction of ammonia and pyruvic acid with the keto acid derivative of the amino acid to give the N-acetyl-amino acid. α-Amino-β-phenylbutyric acid, which is not a naturally occurring substance, was used in these studies. The optical inversion of the unnatural isomer of the α-aminobutyric acid derivative into the acetylated natural enantiomorph *in vivo* is beautifully explained by this theory. The data show, however, that some of the amino acid derivative is oxidized to hippuric acid during the inversion and that a direct acetylation of the natural isomer of the aminobutyric acid derivative, without the preliminary oxidative deamination, cannot be ruled out *a priori* (89).

p-Bromophenyl-*l*-cysteine (129) and S-benzyl-*l*-cysteine (130, 131) are readily acetylated in the rat, rabbit, dog, and man. It was suggested (129) that the acetyl group of the mercapturic acid did not necessarily originate from glutathione as was proposed by Brand & Harris (132), but may have been a result of direct acetylation of the cysteine derivatives. In view of the fact, however, that the acetylation of aromatic amines does not occur in the dog (133), while the acetylation of amino acid derivatives does, it was further suggested that the acetylation mechanisms involving aromatic amines and amino acid derivatives may not be the same (130). The possible formation of S-benzylthiopyruvic acid from S-benzylcysteine as an intermediate in the synthesis of benzylmercapturic acid *in vivo* led to the suggestion that S-benzyl-*d*-cysteine may undergo optical inversion *in vivo* to yield N-acetyl-S-benzyl-*l*-cysteine (130). Similar deductions were made concerning S-benzyl-*d*-homocysteine when it was found that S-benzyl-*l*-homocysteine was readily acetylated in the animal body (134). Du Vigneaud *et al.* (135) have now demonstrated such an inversion of *d*-isomers of S-benzylcysteine and S-benzylhomocysteine in the rat. Significantly, considerably greater yields of the acetylated benzyl derivatives of cysteine and homocysteine were obtained from the urine of rats after the administration of the *l*-isomers of S-benzylcysteine or S-benzylhomocysteine as compared to those isolated after feeding the corresponding *d*-enantiomorphs. In addition, N-acetyl-S-benzyl-*dl*-cysteine was also found in the urine of rats fed S-benzyl-*d*-cysteine. While these results show clearly enough the inversion of one enantio-

morph of an amino acid derivative into its antipode, possibly via the mechanism suggested by Knoop (128), they also demonstrate direct acetylation of S-benzyl-*d*-cysteine in the animal body. Moreover, the greater stability of S-benzyl-*l*-cysteine *in vivo* as compared to that of the *d*-enantiomorph suggests the inference that in the course of acetylation S-benzyl-*d*-cysteine undergoes reactions which lead to its partial destruction and to which S-benzyl-*l*-cysteine is apparently immune (136). Available data do not necessarily support the assumption that N-acetyl-S-benzyl-*l*-cysteine is formed *in vivo* from *d*- or *l*-isomers of S-benzylcysteine via the same mechanism. The possibility of direct acetylation of S-benzyl-*l*-cysteine, as well as of a considerable portion of S-benzyl-*d*-cysteine, and of the natural enantiomorph of α-amino-β-phenylbutyric acid (89) *in vivo* "connotes for the phenomenon of acetylation an entirely different metabolic significance from its role as an intermediary step in reductive amination" (89).

Cysteine (*mercapturic acids*).—Using a quantitative method for the determination of mercapturic acids in urine (137), it was established that *dl*-methionine, *l*-cystine, and *l*-cysteine, but not taurine, increased the extent of the synthesis of *p*-bromophenylmercapturic and *l*-α-naphthalenemercapturic acids in dogs (138) and rats (139). The synthesis of these acids in dogs is not affected by removal of bile from circulation (140), or by feeding the food and the carbocyclic substances at various times of the day (141). The extent of the synthesis of *p*-bromophenylmercapturic acid in the growing dog was shown to be a function of the body weight of the animal (142).

In accord with earlier observations (143), the extent of the synthesis of ethereal sulfates and mercapturic acid from bromobenzene in the dog was found to be affected by the sulfur-containing amino acid make-up of the diet (144). Consideration of the nitrogen partition of the urine of dogs under various dietary conditions (138, 144) led to the conclusion that the cysteine of the mercapturic acids is derived from the tissues, and that the sulfur-containing amino acids of the diet affect the mercapturic acid formation only in so far as they determine the nutritional state of the animal.

Glutathione (145) and *dl*-homocystine (146) promote the growth of cystine-deficient rats and counteract the inhibition of growth in rats produced by the administration of bromobenzene (147) or naphthalene (148). Since no increase was noted in the extent of the synthesis of corresponding mercapturic acids from bromobenzene or naphthalene upon administration of glutathione (139) or *dl*-homocystine (147) to

rats, the stimulation of the growth of rats by these substances was not due to increased detoxication of bromobenzene or naphthalene. Feeding of S-benzylglutathione to rats led to the excretion of benzylmercapturic acid in the urine, indicating the hydrolysis of the glutathione derivative to S-benzylcysteine *in vivo* (149). A decrease in the concentration of glutathione in the tissues of animals which were administered bromobenzene (95) or naphthalene (150) was reported. These observations do not necessarily indicate a direct utilization of tissue glutathione for detoxication purposes, as has been suggested (95). Aside from the fact that many factors and unrelated substances were found to increase or decrease the glutathione concentration of the animal tissues, the available data on the relation of glutathione to mercapturic acid synthesis indicate the probability that the source of cysteine for mercapturic acid and glutathione synthesis *in vivo* may be the same. The formation of mercapturic acids takes precedence over the synthesis of glutathione, as well as the need of the animal for the sulfur-containing amino acids for growth purposes. The dietary sulfur-containing amino acids or glutathione may replace the cysteine lost by the animal for detoxication purposes. However, glutathione may be absorbed as such without the preliminary hydrolysis to free cysteine.

Administration of *p*-bromophenol to dogs (143) and rats (151) yielded an ethereal sulfate in the urine, not *p*-bromophenylmercapturic acid. This was interpreted as indicating that *p*-bromophenol cannot be considered an intermediate in the synthesis of the mercapturic acid from bromobenzene. Young (152) is of the opinion that "results of this type, however, can only be regarded as indicative, for it does not follow that the changes which are undergone by a compound produced in the tissues are invariably the same as those undergone by that compound when it is administered to the animal."

It was demonstrated that benzyl chloride (130, 134), when fed or injected to dogs, rabbits, or rats, yielded in the urine N-acetyl-S-benzyl-*l*-cysteine (benzylmercapturic acid). This is the only mercapturic acid so far isolated, in which the sulfur of cysteine is attached to the side chain and not to the carbon atom of the ring. Benzyl alcohol and benzaldehyde, which may conceivably be formed from benzyl chloride *in vivo,* yielded only hippuric acid (134), as was shown earlier (153), and cannot be considered as intermediates in the synthesis of benzylmercapturic acid from benzyl chloride *in vivo*. The possibility was pointed out that tissue protein may be the common factor in the mechanism of sensitization and detoxication of benzyl chloride in

animals (130, 134), in view of the reports that benzyl chloride induces sensitization in animals (154).

The observation of White & Jackson (155) that bromobenzene inhibits the growth of rats maintained on a relatively low casein diet, has now been extended to include naphthalene (148), phenanthrene (156), cholic acid (157), iodoacetic acid (158), diphenyl (159), methylcholanthrene, 3,4-benzpyrene, pyrene (160), benzyl chloride (131), and "butter yellow" (161). The basic assumption made by White & Jackson (155) in explaining the growth inhibition was that cystine deficiency is induced in the growing animals by administration of these substances, and that this deficiency is due to withdrawal of cysteine for synthesis of the corresponding mercapturic acids. While the synthesis of mercapturic acids was demonstrated for monohalogenated benzenes, naphthalene, anthracene, and benzyl chloride, the validity of the basic assumption with respect to the rest of the substances shown to inhibit the growth of rats, has not been proved. Such a demonstration is awaited with interest, especially in the case of carcinogenic compounds. It should be noted, however, that both carcinogenic and noncarcinogenic substances are effective inhibitors of growth of rats (162), and that the carcinogenic property of a substance may not be directly related to the growth inhibition effect.

Wood & Fieser (163) reported the synthesis of sulfhydryl and cysteine derivatives of certain carcinogenic substances. The cysteine derivatives proved to be unstable even in neutral aqueous medium, yielding the corresponding disulfides. The authors suggested that the disulfides of the carcinogenic substances may still undergo conjugation with cysteine *in vivo* to yield the corresponding mercapturic acids. Since dibenzyl disulfide was oxidized *in vivo* to benzoic acid and was not converted to benzylmercapturic acid, the formation of the hydroxyl or the carboxyl derivative from the disulfides of the carcinogenic substances synthesized by Wood & Fieser appeared to be the more likely possibility (136).

The administration of bromobenzene to animals poisoned by selenium led to elimination of selenium in the urine accompanied by the improvement of the animals. It was suggested that bromobenzene removed tissue selenium possibly as a mercapturic acid (164).

Detoxication mechanism and related topics have been reviewed by several authors (152, 165 to 170).

LITERATURE CITED

1. BERNHARD, K., *Z. physiol. Chem.*, **248**, 256 (1937)
2. FLASCHENTRAGER, B., AND BERNHARD, K., *Z. physiol. Chem.*, **256**, 71 (1938)
3. TORBOLI, A., *Boll. soc. ital. biol. sper.*, **12**, 368 (1937)
4. BERNHARD, K., *Z. physiol. Chem.*, **256**, 49 (1938)
5. BERNHARD, K., *Z. physiol. Chem.*, **256**, 59 (1938)
6. BERNHARD, K., *Z. physiol. Chem.*, **258**, 96 (1939)
7. BERNHARD, K., *Z. physiol. Chem.*, **257**, 54 (1939)
8. DRUMMOND, J. C., AND FINAR, I. L., *Biochem. J.*, **32**, 79 (1938)
9. JAFFÉ, M., *Z. physiol. Chem.*, **62**, 58 (1909)
10. CALLOW, E. H., AND HELE, T. S., *Biochem. J.*, **20**, 598 (1926); STEKOL, J. A., *Proc. Soc. Exptl. Biol. Med.*, **33**, 170 (1935)
11. JORISSEN, W. P., *Natuurw. Tijdschr.*, **19**, 15 (1937)
12. MUELLER, H. K., *Nature*, **132**, 280 (1933); MUELLER, H. K., BUSCHKE, W., GUREWITSCH, A., AND BRUHL, F., *Klin. Wochschr.*, **13**, 20 1934); MUELLER, H. K., *Arch. Augenheilk.*, **109**, 304 (1935)
13. MUELLER, H. K., *Arch. Ophthalmol. (Graefe's)*, **140**, 171 (1939)
14. BERNHEIM, F., BERNHEIM, M. L. C., AND MICHEL, H. O., *J. Pharmacol.*, **61**, 311 (1937)
15. MICHEL, H. O., BERNHEIM, F., AND BERNHEIM, M. L. C., *J. Pharmacol.*, **61**, 321 (1937)
16. MARSHALL, JR., E. K., AND WALZL, E. M., *Bull. Johns Hopkins Hosp.*, **61**, 140 (1937)
17. MEULENGRACHT, E., AND LUNDSTEEN, I., *Folia Haematol.*, **63**, 89 (1939)
18. WENDEL, W. B., WENDEL, N. M., AND COX, W. W., *J. Biol. Chem.*, **131**, 177 (1939)
19. SCUDI, J. V., *Science*, **91**, 486 (1940)
20. SHELSWELL, J., AND WILLIAMS, R. T., *Biochem. J.*, **34**, 528 (1940)
21. JAMES, G. V., *Biochem. J.*, **34**, 640 (1940)
22. JAMES, G. V., *Biochem. J.*, **34**, 636 (1940)
23. ROSENTHAL, S. M., AND BAUER, H., *U.S. Pub. Health Repts.*, **54**, 1880 (1939)
24. SHINN, L. E., MAIN, E. R., AND MELLON, R. R., *Proc. Soc. Exptl. Biol. Med.*, **42**, 736 (1939)
25. MANN, T., AND KEILIN, D., *Nature*, **146**, 164 (1940)
26. DOANE, J. C., BLUMBERG, N., AND TEPLICK, J. G., *Med. Record*, **151**, 439 (1939)
27. SCHNITKER, M. A., *Sulfanilamide, Sulfapyridine and Allied Compounds in Infections* (Oxford University Press, New York, 1940)
28. MARSHALL, JR., E. K., AND LICHTFIELD, JR., J. T., *J. Pharmacol.*, **67**, 454 (1939)
29. POWELL, H. M., AND CHEN, K. K., *J. Pharmacol.*, **67**, 79 (1939)
30. WURM, K., *Deut. med. Wochschr.*, **66**, 43, 73, 102 (1940)
31. KOLMER, J. A., *Arch. Internal Med.*, **65**, 671 (1940)
32. BOYLAND, E., AND LEVI, A., *Chemistry & Industry*, **15**, 446 (1937)
33. DOBRINER, K., RHOADS, C. P., AND LAVIN, G. I., *Proc. Soc. Exptl. Biol. Med.*, **41**, 67 (1939)
34. CHALMERS, J. G., *Biochem. J.*, **32**, 271 (1938); **34**, 678 (1940)

35. NEUFACH, S. A., *Uspekhi Sovrem. Biol.*, **8**, 282 (1938)
36. STROUD, S. W., *Nature,* **146**, 166 (1940)
37. WESTERFELD, W. W., *Biochem. J.*, **34**, 51 (1940)
38. STROUD, S. W., *J. Endocrinol.*, **2**, 55 (1940)
39. GOEBEL, W. F., *J. Biol. Chem.*, **122**, 649 (1937–38)
40. PRYDE, J., AND WILLIAMS, R. T., *Biochem. J.*, **30**, 794 (1936)
41. QUICK, A. J., *Biochem. J.*, **28**, 403 (1934)
42. DI SOMMA, A. A., *J. Biol. Chem.*, **133**, 277 (1940)
43. LEHMANN, G., AND KNOEFEL, P. K., *Am. J. Med. Sci.*, **197**, 639 (1939)
44. WILLIAMS, R. T., *Biochem. J.*, **32**, 1849 (1938)
45. WILLIAMS, R. T., *Biochem. J.*, **34**, 690 (1940)
46. SNAPPER, I., YU, T. F., AND CHIANG, Y. T., *Proc. Soc. Exptl. Biol. Med.*, **44**, 30 (1940)
47. KUHN, R., AND LOW, I., *Z. physiol. Chem.*, **254**, 139 (1938)
48. SCHMID, F., *Compt. rend. soc. biol.*, **123**, 223 (1936)
49. BLISS, JR., A. R., AND GLASS, H. B., *J. Am. Pharm Assoc.*, **29**, 171 (1940)
50. UMEZAWA, S., AND MASAMUNE, H., *J. Biochem. (Japan)*, **28**, 487 (1938)
51. MASAMUNE, H., *J. Biochem. (Japan)*, **25**, 299 (1937)
52. WILLIAMS, R. T., *Biochem. J.*, **32**, 1849 (1938)
53. WILLIAMS, R. T., *Biochem. J.*, **33**, 1519 (1939)
54. MAUGHAN, G. B., EVELYN, K. A., AND BROWNE, J. S. L., *J. Biol. Chem.*, **126**, 567 (1938)
55. MOSOLOWSKI, W., *Biochem. J.*, **34**, 823 (1940)
56. WAGREICH, H., KAMIN, H., AND HARROW, B., *Proc. Soc. Exptl. Biol. Med.*, **43**, 468 (1940)
57. CRISMER, R., *Compt. rend. soc. biol.*, **132**, 481 (1939); **133**, 271 (1940)
58. KAPP, E. M., *J. Biol. Chem.*, **134**, 143 (1940)
59. FISHMAN, W. H., *J. Biol. Chem.*, **127**, 367 (1939)
60. MASAMUNE, H., *J. Biochem. (Japan)*, **19**, 353 (1934)
61. FISHMAN, W. H., *J. Biol. Chem.*, **131**, 225 (1939)
62. ODELL, A. D., SKILL, D. I., AND MARRIAN, G. F., *J. Pharmacol.*, **60**, 420 (1937)
63. OSHIMA, G., *J. Biochem. (Japan)*, **20**, 361 (1934); **23**, 305 (1936)
64. FISHMAN, W. H., *J. Biol. Chem.*, **136**, 229 (1940)
65. HEMINGWAY, A., PRYDE, J., AND WILLIAMS, R. T., *Biochem. J.*, **38**, 136 (1934)
66. SALT, H. B., *Biochem. J.*, **29**, 2705 (1935)
67. BUEDING, E., *Rept. Turkish Soc. Phys. Nat. Sci.*, reprint, 4 (Oct. 8, 1938)
68. LIPSCHITZ, W. L., AND BUEDING, E., *J. Biol. Chem.*, **129**, 333 (1939)
69. BUEDING, E., AND LADEWIG, P., *Proc. Soc. Exptl. Biol. Med.*, **42**, 464 (1939)
70. NAZARYANTZ, B. A., *Ukrain. Biokhem. Zhur.*, **5**, 129 (1932)
71. PETERS, R. A., AND DAVENPORT, H. W., *Ann. Rev. Biochem.*, **7**, 325 (1938); KING, C. G., *Ann. Rev. Biochem.*, **8**, 392 (1939)
72. FRIEMANN, *Arch. Gewerbepath. Gewerbehyg.*, **7**, 278 (1936); BORMANN, G., *Arch. Gewerbepath. Gewerbehyg.*, **8**, 194 (1937); CASTROVILLI, G., *Med. lavoro*, **28**, 106 (1937); *Chimie & industrie*, **39**, 272 (1937); MEYER, A., *Z. Vitaminforsch.*, **6**, 83 (1937); HAGEN, J., *Arch. Gewerbepath. Gewerbehyg.*, **8**, 541 (1938)

73. KOIKE, M., YOKOYAMA, H., AND TANAKA, K., *Osaka Igk. Z.*, **35**, 1705 (1936); LEIBOWITZ, J., AND GUGGENHEIM, K., *Harefuah Med. J.*, **14**, 224 (1938)

74. HOLMES, H. N., CAMPBELL, K., AND AMBERY, E. J., *J. Lab. Clin. Med.*, **24**, 1119 (1939)

75. LEIBOWITZ, J., AND GUGGENHEIM, K., *Z. Vitaminforsch.*, **8**, 8 (1938)

76. BORSETTI, N., *Arch. ist. biochim. ital.*, **10**, 3 (1938)

77. FRIEND, D. G., AND MARQUIS, H. H., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **22**, 239 (1938); CORMIA, F. E., *J. Investigative Dermatol.*, **1**, 199 (1938)

78. RYANG, W. T., *Trans. Soc. Path. Japon.*, **28**, 486 (1938); RYO, G., *J. Chosen Med. Assoc.*, **28**, 1494 (1938)

79. LONGENECKER, H. E., FRICKE, H. H., AND KING, C. G., *J. Biol. Chem.*, **135**, 497 (1940); LONGENECKER, H. E., MUSULIN, R. R., TULLY, 3RD, R. H., AND KING, C. G., *J. Biol. Chem.*, **129**, 445 (1939)

80. ARMSTRONG, E. F., AND ARMSTRONG, K. F., *The Glycosides* (Longmans, Green and Co., London, 1931)

81. MILLER, L. P., *Contrib. Boyce Thompson Inst.*, **9**, 425 (1938); **10**, 139 (1939)

82. MILLER, L. P., *Science*, **92**, 42 (1940); *Contrib. Boyce Thompson Inst.*, **11**, 271 (1940)

83. BORSOOK, H., AND DUBNOFF, J. W., *J. Biol. Chem.*, **132**, 307 (1940)

84. WAELSCH, H., AND BUSZTIN, A., *Z. physiol. Chem.*, **249**, 135 (1937)

85. QUICK, A. J., *J. Biol. Chem.*, **95**, 189 (1932)

86. SCHOENHEIMER, R., RITTENBERG, D., FOX, M., KESTON, A. S., AND RATNER, S., *J. Am. Chem. Soc.*, **59**, 1768 (1937)

87. RITTENBERG, D., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **127**, 329 (1939)

88. WOELSCH, H., AND RITTENBERG, D., *Science*, **90**, 423 (1939)

89. VIGNEAUD, V. DU, COHN, M., BROWN, G. B., IRISH, O. J., SCHOENHEIMER, R., AND RITTENBERG, D., *J. Biol. Chem.*, **131**, 273 (1939)

90. TERROINE, E. F., AND BOY, G., *Arch. intern. pharmacodynamie*, **63**, 300 (1939)

91. VASILIU, H., HUBER, Z., TIMOSENCU, A., ZAIMOV, C., AND COTELEU, V., *Bul. Facultăt. Stiinţe Agr. Chişinău, Comun. Lab. Chim. Agr.*, **2**, 27 (1938); VASILIU, H., TIMOSENCU, A., ZAIMOV, C., AND COTELEU, V., *Bul. Facultăt Stiinţe Agr. Chişinău, Comun. Lab. Chim. Agr.*, **3**, 77 (1940)

92. GRIFFITH, W. H., *J. Biol. Chem.*, **64**, 401 (1925)

93. ABBOTT, JR., L. D., AND LEWIS, H. B., *J. Biol. Chem.*, **131**, 479 (1939)

94. BLOCH, K., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **135**, 99 (1940)

95. WAELSCH, H., *Arch. exptl. Path. Pharmakol.*, **156**, 356 (1933); WAELSCH, H., AND WEINBERGER, E., *Arch. exptl. Path. Pharmakol.*, **156**, 370 (1933)

96. HARROW, B., AND SHERWIN, C. P., *Ann. Rev. Biochem.*, **4**, 263 (1935)

97. QUICK, A. J., *Am. J. Clin. Path.*, **10**, 222 (1940)

98. SNELL, A. M., AND PLUNKETT, J. E., *Am. J. Digestive Diseases Nutrition*, **2**, 716 (1936)

99. KOHLSTAEDT, K. G., AND HELMER, O. M., *Am. J. Digestive Diseases Nutrition*, **3**, 501 (1936)

100. FOUTS, P. J., HEIMER, O. M., AND ZERFAS, L. G., *Am. J. Med. Sci.,* **193**, 647 (1937)
101. BARTELS, E. C., AND PERKIN, H. J., *New Engl. J. Med.,* **216**, 1051 (1937)
102. YARDUMIAN, K., AND ROSENTHAL, P. J., *J. Lab. Clin. Med.,* **22**, 1046 (1937)
103. QUASTEL, J. H., AND WALES, W. T., *Lancet,* **II**, 301 (1938)
104. STROM-OLSEN, R., GREVILLE, G. D., AND LEUNON, R. W., *Lancet,* **II**, 995 (1938)
105. LINDEBOOM, G. A., *Acta Med. Scand.,* **99**, 147 (1939)
106. FINKELMAN, I., HORRA, J., SHERMAN, I., AND HORWITT, M. K., *Am. J. Psychiat.,* **96**, 951 (1940)
107. WILSON, S. J., *J. Lab. Clin. Med.,* **25**, 1139 (1940)
108. WILLIAMS, R. T., *Biochem. J.,* **32**, 878 (1938)
109. MARENZI, A. D., *Rev. soc. argentina biol.,* **12**, 205 (1936)
110. BARAC, G., *Compt. rend. soc. biol.,* **124**, 264 (1937)
111. BARAC, G., *Compt. rend. soc. biol.,* **126**, 2 (1937)
112. BLASCHKO, H., RICHTER, D., AND SCHLOSSMANN, H., *J. Physiol.,* **89**, 39 (1937)
113. RICHTER, D., *Biochem. J.,* **32**, 1763 (1938)
114. RICHTER, D., *J. Physiol.,* **98**, 361 (1940)
115. LOEPER, M., AND PARROD, J., *Bull. soc. chim. biol.,* **20**, 1117 (1938)
116. LOEPER, M., LOEPER, J., LEMAIRE, A., COTTET, J., AND PARROD, J., *Compt. rend. soc. biol.,* **128**, 1050 (1938)
117. WILEY, F. H., *J. Biol. Chem.,* **124**, 627 (1938)
118. HUEPER, W. C., WILEY, F. H., WOLFE, H. D., RANTA, K. E., LEMING, M. F., AND BLOOD, F. R., *J. Ind. Hyg. Toxicol.,* **20**, 46 (1938)
119. CLEARY, R. V., MAIER, J., AND HITCHINGS, G. H., *J. Biol. Chem.,* **127**, 403 (1939)
120. COLLIER, H. B., *Can. J. Research, D,* **18**, 272 (1940)
121. SWALLES, W. E., *Can. J. Research, D,* **18**, 266 (1940); SWALLES, W. E., AND COLLIER, H. B., *Can. J. Research, D,* **18**, 279 (1940)
122. KLEIN, J. R., AND HARRIS, J. S., *J. Biol. Chem.,* **124**, 613 (1938)
123. STEWART, J. D., ROURKE, G. M., AND ALLEN, J. G., *Surgery,* **5**, 232 (1932)
124. JAMES, G. V., *Biochem. J.,* **33**, 1688 (1939)
125. MARSHALL, JR., E. K., CUTTING, W. C., AND EMERSON, JR., K., *J. Am. Med. Assoc.,* **110**, 252 (1938)
126. KOHL, M. F. F., AND FLYNN, L. M., *Proc. Soc. Exptl. Biol. Med.,* **44**, 455 (1930)
127. VIGNEAUD, V. DU, AND IRISH, O. J., *J. Biol. Chem.,* **122**, 349 (1938)
128. KNOOP, F., AND BLANCO, J. G., *Z. physiol. Chem.,* **146**, 267 (1925)
129. STEKOL, J. A., *J. Biol. Chem.,* **122**, 333 (1938)
130. STEKOL, J. A., *J. Biol. Chem.,* **124**, 129 (1938)
131. STEKOL, J. A., *Proc. Am. Chem. Soc.* (100th meeting, Detroit, 1940)
132. BRAND, E., AND HARRIS, M. M., *Science,* **77**, 589 (1933)
133. COHN, R., *Z. physiol. Chem.,* **17**, 274 (1893); ELLINGER, A., AND HENSEL, M., *Z. physiol. Chem.,* **41**, 21 (1914); CERECEDO, L. R., AND SHERWIN, C. P., *J. Biol. Chem.,* **58**, 215 (1923–24); **62**, 217 (1924–25); MUENZEN, J. B., CERECEDO, L. R., AND SHERWIN, C. P., *J. Biol. Chem.,* **68**, 503 (1926)

134. STEKOL, J. A., *J. Biol. Chem.*, **128**, 199 (1939)
135. VIGNEAUD, V., DU, WOOD, J. L., AND IRISH, O. J., *J. Biol. Chem.*, **129**, 171 (1939)
136. STEKOL, J. A., *Proc. Soc. Exptl. Biol. Med.*, **45**, 693 (1940)
137. STEKOL, J. A., *J. Biol. Chem.*, **113**, 279 (1936)
138. STEKOL, J. A., *J. Biol. Chem.*, **117**, 147 (1937)
139. STEKOL, J. A., *J. Biol. Chem.*, **122**, 333 (1938)
140. STEKOL, J. A., AND MANN, F. C., *J. Biol. Chem.*, **117**, 619 (1937)
141. STEKOL, J. A., *J. Biol. Chem.*, **118**, 155 (1937)
142. STEKOL, J. A., *J. Biol. Chem.*, **121**, 93 (1937)
143. WHITE, A., AND LEWIS, H. B., *J. Biol. Chem.*, **98**, 607 (1932)
144. STEKOL, J. A., *Proc. Soc. Exptl. Biol. Med.*, **35**, 623 (1937)
145. DYER, H. M., AND VIGNEAUD, V., DU, *J. Biol. Chem.*, **115**, 543 (1936)
146. VIGNEAUD, V., DU, DYER, H. M., AND HARMON, J., *J. Biol. Chem.*, **101**, 719 (1933)
147. STEKOL, J. A., *J. Biol. Chem.*, **133**, xcviii (1940)
148. STEKOL, J. A., *J. Biol. Chem.*, **127**, 131 (1939)
149. STEKOL, J. A., *Proc. Soc. Exptl. Biol. Med.*, **43**, 108 (1940)
150. NAKASHIMA, T., *J. Biochem. (Japan)*, **19**, 281 (1934)
151. STEKOL, J. A., AND DASH, H. H., *Proc. Soc. Exptl. Biol. Med.*, **40**, 261 (1939)
152. YOUNG, L., *Physiol. Rev.*, **19**, 323 (1939)
153. SIEBER, N., AND SMIRNOW, A., *Monatsh.*, **8**, 88 (1887); COHN, R., *Z. physiol. Chem.*, **17**, 274 (1893); ROEHMANN, F., *Biochemie*, p. 413 (Berlin, 1908); SHERWIN, C. P., AND HYNES, W. A., *J. Biol. Chem.*, **47**, 297 (1921)
154. LANDSTEINER, K., AND JACOBS, J., *J. Exptl. Med.*, **64**, 625 (1936)
155. WHITE, A., AND JACKSON, R. W., *J. Biol. Chem.*, **111**, 507 (1935)
156. STEKOL, J. A., *Proc. Soc. Exptl. Biol. Med.*, **42**, 328 (1939)
157. WHITE, A., *J. Biol. Chem.*, **112**, 503 (1935–36)
158. STEVENSON, E. S., AND WHITE, A., *J. Biol. Chem.*, **134**, 709 (1940)
159. WEST, H. D., *Proc. Soc. Exptl. Biol. Med.*, **43**, 373 (1940)
160. WHITE, J., AND WHITE, A., *J. Biol. Chem.*, **131**, 149 (1939)
161. WHITE, J., *J. Biol. Chem.*, **133**, cviii (1940)
162. WHITE, A., AND WHITE, J., *Yale J. Biol. Med.*, **12**, 427 (1940)
163. WOOD, J. L., AND FIESER, L. F., *J. Am. Chem. Soc.*, **62**, 2674 (1940)
164. MOXON, A. L., SCHAEFFER, A. E., LARDY, H. A., DuBois, K. P., AND OLSON, O. E., *J. Biol. Chem.*, **132**, 785 (1940)
165. TSU-YIH-KING, *Chemistry (China)*, **4**, 273 (1937)
166. FIESER, L. F., *Am. J. Cancer*, **34**, 37 (1938)
167. SCHÖBERL, A., *Angew. Chem.*, **53**, 227 (1940)
168. FILDES, P., *Lancet*, **I**, 955 (1940)
169. OETTINGEN, W. F. VON, *U.S. Pub. Health Bull.*, No. 255, 1 (1940)
170. PICK, E. P., *J. Mt. Sinai Hosp.*, **7**, 99 (1940)

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HORMONES

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A review of the work on hormones for 1940 has been made under two handicaps: first, the impossibility of securing a number of articles published in foreign journals, and second, the necessity to omit mention of many important contributions. Recognition of the merit of the work has not been the only factor. Many outstanding investigations have not been included simply because of lack of space.

THYROXINE

Synthesis of Thyroxine "in vitro"

Iodized serum protein administered to patients with myxedema relieves the symptoms of hypothyroidism and increases the basal metabolic rate. Treatment with alkali or enzymes permits the separation of the iodized protein into two fractions: one, insoluble in acid, contains the physiologic activity and the other, soluble in acid, is similar to the diiodotyrosine fraction of thyroglobulin. The fraction insoluble in acid contains material which resembles iodothyronine, but thyroxine was not isolated (1).

Other work on the iodization of proteins has now furnished convincing evidence in regard to the nature of the compound which is responsible for the increase in basal metabolic rate. If casein or other protein is treated in an ammoniacal solution or in acetic acid with iodine at 0° C., little if any material with thyroid activity is produced, but if the protein is iodized at a higher temperature significant amounts of material with thyroid activity are formed. When the iodized protein is hydrolyzed and fractionated, monoiodotyrosine, diiodotyrosine, and finally thyroxine itself can be separated in pure crystalline form (2).

Since four atoms of iodine cannot be added to thyronine the only possible course by which thyroxine could be prepared from casein is through diiodotyrosine. Evidence that this is the mechanism by which thyroxine is synthesized has been furnished by the following experiment. The sodium salt of diiodotyrosine in water solution at a pH of about 8 was held at a slightly elevated temperature for some days. The solution was then made acid and from the acid-

insoluble fraction pure crystalline thyroxine was separated and identified (3).

Since the amount of thyroxine which had been formed was only a small fraction of the weight of diiodotyrosine there still remained the possibility that some impurity was present in the diiodotyrosine which was the source of the thyroxine. In order to remove this possibility the experiment has now been carried out under still more exacting conditions. Tyrosine was synthesized and was then converted into d,l-diiodotyrosine. When this material was dissolved in sodium hydroxide and maintained at a pH of 8.8 and a temperature of 70° C. for fourteen days, thyroxine was formed from the diiodotyrosine. It was precipitated with acid, purified through treatment with butyl alcohol and separated as the potassium salt (4).

Diiodotyrosine has but little if any effect on the basal metabolic rate but the possibility must now be recognized that diiodotyrosine may slowly be converted into thyroxine in the animal organism even in the absence of the thyroid gland. No evidence has been presented which shows that diiodotyrosine can enhance the effect of thyroxine or exert a synergistic effect.

RESPONSE TO THYROXINE AND THYROGLOBULIN

The discrepancy between crystalline d,l-thyroxine and l-thyroxine, as it occurs in desiccated thyroid, when the response on the basal metabolic rate is measured, is explained in all probability by the inactivity of the dextro form (5) and results from experiments *in vitro* indicate that thyroxine does not exert its maximal effect unless it is combined with protein. At low levels of metabolism thyroglobulin stimulates consumption of oxygen by guinea pig tissues but there is a "ceiling" above which the thyroglobulin cannot further elevate the uptake of oxygen. The addition of thyroxine generally does not increase the uptake of oxygen, but in contact with serum thyroxine may be made physiologically active *in vitro* (6).

Although previous work had indicated the presence of thyroglobulin in serum, a more recent investigation failed to obtain evidence for the presence of thyroglobulin in systemic or thyroid blood before or after injection of enough thyroxine or thyrotropic hormone to produce a marked rise in metabolism (7).

SOME PHYSIOLOGIC EFFECTS OF THYROXINE

Stimulation of the thyroid gland through administration of thyrotropic hormone interrupts gestation in pregnant rabbits. The most

probable mode of action is through stimulation of the thyroid gland of the maternal animal with liberation of an excessive amount of thyroxine, which by its toxic action kills the fetus (8).

A single injection of thyroxine is followed by alterations in the chemical composition and the histologic structure of the rat liver, which vary with the time elapsed after the injection. There is a loss of glycogen in the liver and in the entire animal. Forty-eight hours after the injection there is an increase in glycogen. In the first period after injection the protein in the liver increases, then decreases, but the concentration remains above normal. Sugar and lactic acid in the liver and blood remain unchanged during the first period but later increase simultaneously with the glycogen. The fresh and dry weights of the liver also increase. The loss in glycogen, and the increase in the protein and the size of the liver all begin before the consumption of oxygen increases. The changes in the liver revealed by histologic examination suggest that a growth of the liver takes place under the conditions of the experiment (9). The administration of thyroxine diminishes the phosphagen of the heart of a resting rat, and the glycogen, phosphagen, and adenylpyrophosphate of the heart after exercise (10). Thyroxine also produces sustained creatinuria and prevents deposition of glycogen in the liver even after administration of glucose and insulin (11).

The rise in the basal consumption of oxygen produced by thyroxine is decreased in male cats treated at the same time with carotene although the loss in weight is not significantly different (12). Sulfanilamide and phenobarbital reduce the degree of experimental hyperthyroidism (13), and the administration of estradiol or diethylstilbestrol to thyroidectomized rats that had received desiccated thyroid reduced by a half the time required for the basal metabolic rate to return to the former low level (14, 15). In the presence of a state of hyperthyroidism, galactose, administered orally, is absorbed from the intestine three times faster than normal (16).

The increased basal metabolic rate produced by the administration of desiccated thyroid is associated with an increase in the activity of the d-amino acid oxidase in rat liver. This enzyme is composed of a specific protein and a flavin-adenine dinucleotide; the increase in activity of the oxidase is probably due to an increase in the concentration of its protein component and is not due to a change in the nature or concentration of the flavin component (17).

Further evidence has been secured of the striking influence of

Other steroid derivatives which do not have the α-ketol structure possess some physiologic activity simulating an extract of the adrenal cortex. Strophanthin protects normal animals against the toxic action of potassium, lowers the level of potassium in the plasma, and prolongs life in cats deprived of their adrenals (40). In the mouse, however, strophanthin is ineffective (41).

Assay

In a method of assay for extracts of the adrenal cortex based on survival and growth, 1 rat unit is defined as the minimum daily dose of hormone sufficient to protect at least 80 per cent of the rats and produce an average growth of at least 20 gm. for a twenty-day period in four-week-old male rats of 50 to 60 gm. weight. Oral administration gives the same results as parenteral administration for extracts of the adrenal cortex, corticosterone, and dehydrocorticosterone; desoxycorticosterone acetate assayed less than 1 rat unit per milligram when administered orally but it assayed 35 rat units per milligram when given parenterally (42).

For the assay of cortical extracts in the dog the experimental condition is reported to be more significant than changes in the concentration of the constituents of the blood. Variations in water balance are of little value but the changes in the appetite and temperature provide a satisfactory criterion. The choice of the period of assay is not important; a period of four daily injections is sufficient for any one concentration of the extract (43, 44, 45).

Results from this laboratory on the assay of extracts of the adrenal cortex indicate that on a diet low in potassium an increase in the concentration of urea in the blood of adrenalectomized dogs precedes any change in the appetite or condition of the dog and provides the most satisfactory criterion.

The Adrenal Cortex and Mineral Metabolism

Adrenalectomy in all animals invariably produces a disturbance in the distribution and excretion of sodium, potassium, and chloride. The fundamental importance of these changes was emphasized some years ago by the observations that adrenalectomized animals could be maintained indefinitely without an extract from the adrenal cortex, provided a high intake of sodium chloride and excess sodium ion in the form of bicarbonate or citrate and a low intake of potassium were given. These results have been confirmed in many laboratories. From

650 to 940 mg. of sodium chloride daily is optimal for an adult adrenalectomized rat; 339 mg. is not sufficient for growth or survival and 1200 mg. a day is injurious (46, 47, 48, 49).

Permeability of membranes.—In the adrenalectomized dog withdrawal of the administration of an extract of the adrenal cortex produces a marked decrease in the rate of absorption of sodium, potassium, and chloride from loops of the ileum. This trend is reversed by the administration of the extract. When the animals are maintained on a diet high in sodium and low in potassium the rate of absorption of sodium tends to decline more than that of potassium. Sodium may be excreted into the intestine in relatively large quantity although the potassium ion is still being absorbed. In spite of the fact that dogs maintained with a high sodium and low potassium intake have essentially normal values for the constituents in the blood and although they are objectively in excellent health the behavior of the intestine is not normal if the cortical hormone is withheld (50).

Changes in the serum indicate that the acidosis present in adrenalectomized dogs results from a relatively greater loss of sodium over chloride, an extension of base bound by protein, and an increase in undetermined acid value. The administration of cortical extract in spite of a relative constancy of water in the serum produces a gain in the concentration of sodium and chloride in the serum, some increase in pH and bicarbonate content, and a decrease in the concentration of potassium. The increase in concentrations of sodium and chloride in the serum may be interpreted as being due to an entrance of interstitial fluid into the circulation followed by an excretion of water and some electrolytes with a partial retention of sodium chloride. The loss of sodium from the blood cells is followed by a gain in sodium after administration of cortical extract, but throughout these changes the diffusible ions are influenced by the nondiffusible ions according to the Gibbs-Donnan law (51).

The outstanding changes in the skeletal muscles in adrenal deficiency are a loss of sodium and a gain in water and a tendency for the concentration of chloride ion to decrease and the potassium ion to increase. A decided improvement in the condition of the animal after treatment with an extract of the adrenal cortex alone may not be accompanied by a marked change in the concentration of electrolytes in the skeletal muscle (52).

The permeability of membranes has been studied through the intraperitoneal injection of glucose and sucrose. In the normal rat this

is followed by 100 per cent recovery. Adrenalectomized rats soon are prostrated and the mortality rate is of the order of 80 per cent. The volume of ascitic fluid after adrenalectomy is much less than in the normal rat. There is a marked hemoconcentration which is not relieved despite the absorption of fluid from the peritoneal cavity. Apparently about 25 per cent of the extracellular fluid may be blocked from active participation in the circulation of fluid and electrolytes by stasis of the capillaries (53). The administration of desoxycorticosterone acetate or progesterone results in a marked increase in the rate of entrance of sodium and chloride into a solution of 5.5 per cent glucose introduced into the peritoneal cavity. If samples of fluid are removed five, ten, and fifteen minutes after completion of the intraperitoneal instillation the concentrations of sodium and chloride may be 100 per cent greater than in untreated animals. After thirty minutes there is no significant difference in the samples (54).

An inflammatory exudate induces an increase in the permeability of the capillaries which is manifested by the accumulation from the circulation of a blue dye in the cutaneous area previously injected with the exudate. This effect of the exudate on the permeability of the capillaries is inhibited wholly or in part by the presence of an extract from the adrenal cortex which is added to the exudate when it is injected; or the hormone may be injected separately several minutes or hours before the exudate (55).

The excretion of bromide from patients intoxicated with this ion is materially hastened when sodium chloride and cortical hormone are given. Toxic levels of sodium bromide can be reduced to nontoxic levels in from two to four days (56).

The influence of the adrenal cortex on metabolism of potassium.— The resistance of adrenalectomized mice to the toxic effects of potassium may be raised above that of normal animals by appropriate doses of desoxycorticosterone acetate. For a dose of less than 0.5 mg. a linear relationship exists between the percentage of animals surviving the injection of potassium chloride and the logarithm of the dose of hormone (57).

The intravenous injection of a solution of glucose in the dog decreases the content of potassium in the serum. A similar reaction has now been shown during the fermentation of glucose by yeast. If glucose is added to a suspension of yeast cells in water the concentration of potassium in the water rapidly decreases. When fermentation starts, the potassium is liberated and reaches even higher levels in

solution than before. The movement of potassium is apparently concerned with the production of phosphoric acid esters (58), but it remains for the future to ascertain whether potassium activates the process of phosphorylation or the breakdown of hexosephosphate (59).

If the sciatic artery of a toad is perfused with potassium-free Ringer's solution which contains acetylcholine at a concentration of 1×10^{-6} or more, the contraction of the muscle is accompanied by an increase in the concentration of potassium in the fluid flowing from the vein. If the muscle is denervated, ten times more potassium is liberated than from normal muscle. On the other hand, the curarized muscle does not react to acetylcholine and does not liberate potassium (60). The liberation of potassium from the muscle by acetylcholine occurs in the cat as well as in the toad. Electrical stimulation of muscle also liberates potassium, but after adrenalectomy no potassium whatever is liberated by the action of acetylcholine (in nine experiments on four cats, but the degree of adrenal deficiency was not stated). Similarly the electrical stimulation of muscle does not show an increase in potassium in adrenalectomized cats (61).

The response measured by the Everse-deFremery method to a tetanic stimulation of the skeletal muscle of adult male rats cannot be related to the amount of potassium in the muscles or serum. The results indicate that the myasthenia noted in adrenal insufficiency is not due specifically to an increase in the content of potassium in skeletal muscle (62).

Some Metabolic and Physiologic Processes Affected by the Adrenal Cortex

The general decrease in metabolic activity subsequent to adrenalectomy is associated with a reduction in the rate of utilization of acetone bodies and it is probable that a similar reduction in the rate of formation of acetone bodies occurs (63, 64). Adrenalectomy brings about a diminution of the histaminase content of the lung but the histaminase activity can be restored to normal limits by the administration of an extract of the adrenal cortex (65).

In the kidney of adrenalectomized rats there is a marked decrease in the uptake of oxygen *in vitro,* which probably is due to the high respiratory rate of this tissue. A clear demonstration of the direct effect of the absence of the adrenal hormones on the metabolism of tissue other than the kidney cannot be shown until sufficient time has elapsed for the specific deficiency to be manifest (66). The rate of

production of ammonia in kidney slices from adrenalectomized rats is about 18 per cent less than normal. Kidney slices from adrenalectomized rats have a marked disability to deal as rapidly as normal kidney slices with glutamic, α-ketoglutaric, and pyruvic acids and with *d,l*-alanine (67).

Capacity for vigorous muscular activity.—Demedullation of the adrenal does not modify the time required for rats to swim to exhaustion when handicapped by weighting (68), but the capacity of rats for continued work measured by the stimulation of the gastrocnemius muscle is reduced by the removal of one adrenal, and the removal of a portion of the remaining gland further reduces the total amount of work performed (69, 70). Adrenalectomized rats maintained on a diet high in potassium have a high mortality rate and a low capacity for work. Adrenalectomized rats maintained on a diet high in sodium chloride and low in potassium not only survive but are able to perform more work than adrenalectomized animals with a high intake of potassium. However, the work performance of all the adrenalectomized animals is very small when compared to that of normal animals (71).

The injection of pitressin into dogs some months after adrenalectomy is followed by contraction of the pulse wave, slowing of the pulse rate, and a gradual drop in blood pressure to as low as 30 mm., with a gradual return to normal blood pressure (72).

PHYSIOLOGIC EFFECTS PRODUCED BY THE SEPARATED HORMONES OF THE ADRENAL CORTEX

Separation of an extract of the adrenal cortex into many crystalline compounds and an amorphous fraction has provided the opportunity to study the physiologic response not only of the entire extract but of the individual constituents. Differences in the physiologic effects of some of the crystalline compounds of the adrenal cortex become evident when the compounds are tested with the following criteria: (*a*) the maintenance of adrenalectomized dogs; (*b*) the capacity for work, measured by the length of time an adrenalectomized rat can swim; and (*c*) the response of muscle to short stimulation (73).

A fractionation based on distribution between water and ether has been shown to divide the total extract into two parts, one of which will maintain adrenalectomized animals without the retention of sodium, whereas the other fraction has a more marked effect on the retention of the sodium ion (74, 75).

Different effects of these two fractions are also shown by their influence of the survival time of adrenalectomized-nephrectomized rats and on the resistance of normal fasted mice to insulin (76, 77).

It is difficult to compare these results with those obtained with the crystalline compounds. Sharply separated qualitative effects are produced by the hormones of the adrenal cortex. These effects are shown on the distribution of electrolytes and renal function, somatic growth, atrophy of the thymus and adrenal glands, carbohydrate metabolism, resistance to stress such as that produced by insulin, tetanic stimulation of muscle, toxins, and cold (78).

Distribution of electrolytes and renal function.—Desoxycorticosterone and its acetate have the greatest effect on the retention of sodium and suppression of the concentration of potassium in the serum (79, 80, 81). In contrast to this the amorphous fraction, which is obtained by the removal of all crystalline material from the extract of the adrenal, does not suppress the concentration of potassium or raise the concentration of sodium in the serum beyond normal limits even when it is administered in large amounts. The amorphous fraction, however, has by far the most marked influence on renal function when compared with the crystalline hormones on a weight basis. The daily dose for a dog is less than 2 µg. per kilogram of body weight. The daily dose of corticosterone on this basis is about 100 µg. (80).

Somatic growth.—In the growing rat, corticosterone, its acetate, and compound E (17-hydroxy-11-dehydrocorticosterone) retard growth. With compound E there may be a loss of weight. On the other hand, desoxycorticosterone and its acetate maintain a growth rate which is almost normal. The amorphous fraction from the adrenal cortex in the amounts given did not produce as rapid growth as did desoxycorticosterone; neither was there as great retardation as with corticosterone and related compounds (80, 82, 83). Not only does compound E produce a retardation of growth measured by the body weight, but also the growth of the long bones is retarded (82). That these observations may be related to the phosphatase content which is depressed by compound E in the epiphysis of the bones remains a possibility (84). The influence of adrenalectomy on the growth of bones for nine days following the operation is insignificant (85).

The effect of the adrenal hormones on the weight of the thymus and adrenal glands of normal animals.—Closely related to the influence of the adrenal hormones on growth is their effect on the weight of

the thymus and adrenal glands of normal rats. The marked effect on the atrophy of the adrenal gland is confined to corticosterone and related compounds. The administration of desoxycorticosterone in quantities comparable to the amounts of corticosterone used does not produce significant atrophy of the thymus (80). With the injection of 10 mg. of desoxycorticosterone a day, atrophy of the thymus is produced (83), but the injection of such large amounts of desoxy-corticosterone may result in the formation of progesterone (86, 87) or degradation products with androgenic activity and these in turn may produce the atrophy.

Carbohydrate metabolism.—Some of the hormones of the adrenal cortex exert a marked effect on the deposition of glycogen in the liver. This can be shown even *in vitro* by the perfusion of cat liver with Ringer's solution to which 5 per cent glucose and gum acacia and an extract of the adrenal cortex have been added. There was a rise of more than 80 per cent in the content of glycogen after the liver had been perfused for ten minutes. Perfusion for an equal interval with a solution which was the same except for the absence of extract of the adrenal cortex decreased the glycogen of the liver 12 per cent (88).

It has now been clearly shown that the influence of an extract of the adrenal cortex on the deposition of glycogen in the liver (89) is not produced by the amorphous fraction or by desoxycorticosterone, but is brought about by corticosterone and closely related compounds which have an atom of oxygen on carbon 11 (78, 90, 91). These compounds also decrease the rate of oxidation of glucose (90, 92) but after complete hepatectomy an extract of the adrenal cortex does not appear to inhibit the utilization of circulating sugar (93). In addition to the suppression of the utilization of glucose, corticosterone and related compounds increase the rate of the production of glucose from protein (90, 92).

Gluconeogenesis.—The daily administration of 3 mg. of corticosterone to normal rats does not produce glycosuria. The glycogen content of the liver is increased but the catabolism of protein does not proceed unchecked and the formation of carbohydrate does not approach a rate sufficiently high to bring about the loss of glucose through the kidney. The administration of corticosterone to fasted, partially depancreatized rats does not cause an excretion of glucose in the urine; the insulin available is sufficient to control the metabolism of glucose formed from endogenous protein and to prevent its excretion. When, however, corticosterone or related compounds are

given to partially depancreatized rats that receive food, the small amount of insulin available is not sufficient to check gluconeogenesis; sugar is excreted and a negative nitrogen balance is produced (90, 94).

Corticosterone and related compounds increase gluconeogenesis in rats with phlorhizin diabetes even more than in partially depancreatized rats. The excretion of glucose in phlorhizinized rats is reduced to a low value by removal of the adrenal glands, but corticosterone and related compounds will fully restore gluconeogenesis to the high rate of the normal animal (95, 96).

The influence of the hormones of the adrenal cortex on gluconeogenesis in phlorhizin diabetes is not only quantitatively different but there is a qualitative effect. Desoxycorticosterone permits the production of about two thirds as much glucose as corticosterone or compound E; the amorphous fraction from the adrenal cortex has much less effect but neither desoxycorticosterone nor the amorphous fraction prevents convulsions or increases the survival time (95).

In thyroidectomized rats treated with an excess of corticosterone and related compounds, gluconeogenesis is limited by the available amount of thyroxine, and in the presence of an excess of thyroxine, formation of glucose from protein is limited by the available amount of corticosterone and related compounds (97). In hypophysectomized rats the rate of gluconeogenesis is not fully restored by the administration of compound E but is brought back to normal if thyrotropic hormone or thyroxine is administered simultaneously with the hormone of the adrenal cortex (98).

The anti-insulin effect of corticosterone and related compounds.—Corticosterone and related compounds with an oxygen on carbon 11 prevent convulsions in normal fasted mice injected with insulin (91). The antagonistic effect of this group of compounds on insulin is also shown by the protection against convulsions in phlorhizinized, adrenalectomized rats. This protection is not due to an increase in the blood sugar of these animals but appears to be due to a specific antagonism to insulin. Desoxycorticosterone and the amorphous fraction do not have such an effect under these conditions (95). Strophanthin exerts an anti-insulin effect but whether the mechanism of this protection with the cardiac glucoside is the same as with corticosterone has not been shown (40).

Resistance to stress. Tetanic stimulation.—Stimulation of muscle is utilized in the Everse-deFremery test to show the influence of the cortical hormones on the response during the first few seconds of

stimulation and in the Ingle test to show the influence of these hormones on prolonged stimulation which may last over several days. For short stimulation desoxycorticosterone is one of the most active of the hormones of the adrenal cortex. Corticosterone and related compounds are much less active (78).

During long stimulation the greatest effect is produced by those compounds which have an atom of oxygen on carbon 11. Desoxycorticosterone produces little if any effect; however, the administration of a mixture of desoxycorticosterone and compound E increases the efficiency of muscle more than an equal quantity of either compound alone. Progesterone, ethinyl testosterone, testosterone and its propionate, estradiol benzoate, and diethylstilbestrol are without effect. Reduction of the ethylenic bond Δ^4 in compound E destroys its influence in this test (99, 100).

Toxins, shock, trauma, cold.—An extract of the adrenal cortex administered to adrenalectomized rats increases the resistance to the toxic effects normally produced by the injection of typhoid vaccine. Desoxycorticosterone does not provide protection against typhoid vaccine (101), but in mice it does increase the resistance to certain pathogenic bacteria (102).

In the prevention and treatment of shock produced by histamine in rats and mice, parenteral administration of desoxycorticosterone combined with sodium chloride is more effective than the use of either one alone. The prophylactic effect of this combined treatment in raising the resistance of rats and mice to shock caused by histamine is more pronounced than the therapeutic response to the administration of histamine (103).

Adrenalectomized animals treated with an extract of the adrenal cortex are relatively resistant to noxious agents and respond with characteristic antishock phenomena. Corticosterone is very effective in combating shock caused by surgical trauma and other means. Under these same conditions desoxycorticosterone is ineffective (104, 105). The marked fall in volume of the plasma observed in dogs subjected to continuous distention of the small intestine (106) and the decrease in the volume of the plasma in patients following anesthesia are prevented by desoxycorticosterone (107).

Some years ago it was shown that adrenalectomized rats cannot survive in a low environmental temperature. The administration of extracts of the adrenal cortex or the purified crystalline hormones will permit such animals to survive indefinitely. For this action the com-

pounds which affect carbohydrate metabolism and have an oxygen on carbon 11 and the amorphous fraction which does not have any effect on carbohydrate metabolism are about equally effective. This suggests that the essential effect is produced through a control of the distribution of electrolytes and water and maintenance of a normal volume of the blood (78).

THE RELATION BETWEEN THE ADRENAL AND THE PITUITARY GLAND

The close relation between the adrenal and the pituitary gland has been demonstrated by the relief of the diabetic state either by hypophysectomy or by adrenalectomy (90, 108). In the fasted hypophysectomized, depancreatized rat cortical extract is diabetogenic. On the other hand, the influence of an extract of the anterior pituitary indicates that not all activity is mediated through the adrenal cortex, and, in addition, there is synergism between the two hormones.

The synergistic action between the extract of the pituitary and that of the adrenal glands is so striking that a dose of cortical hormone too small to be effective by itself is made fully effective if the two extracts are injected simultaneously. In the absence of the extract from the anterior pituitary gland, corticosterone increases the amount of glycogen in the liver at the expense of oxidation but it does not affect the glycogen in the muscle. The injection of the extract of the anterior pituitary maintains a normal level of glycogen in the muscle during fasting in hypophysectomized rats, without the simultaneous injection of corticosterone. These results leave no doubt that for the maximal influence of the cortical hormone the presence of one or more hormones from the anterior pituitary is required. It also seems probable that the hormones from the anterior pituitary gland cannot exert their maximal effect on carbohydrate metabolism in the absence of the hormones elaborated by the adrenal cortex (109).

THE HORMONES OF THE ADRENAL CORTEX IN CLINICAL MEDICINE

The results which have been obtained in a study of the physiologic effects of the hormones of the adrenal cortex have been applied in clinical medicine with striking success. A large number of patients with Addison's disease have been treated with desoxycorticosterone, and in most cases the distressing symptoms of adrenal cortical deficiency were relieved (110 to 118).

The effect of desoxycorticosterone acetate on the concentration of potassium in the serum (79, 80) suggested a reinvestigation of the

use of potassium salts for patients with Addison's disease who are treated with desoxycorticosterone acetate. Patients with Addison's disease are benefited by a diet high in sodium and low in potassium content. When desoxycorticosterone acetate is administered a serious condition, sometimes associated with edema, is brought about by continuation of this diet. The intake of more potassium and less sodium is followed by prompt relief of the edema and marked improvement in the clinical condition (119, 120, 121). In some cases desoxycorticosterone increases the blood pressure to hypertensive levels. Reduction of the intake of sodium chloride and the use of small daily doses of desoxycorticosterone will lower the blood pressure and control this undesirable effect (118).

Some patients with Addison's disease have exhibited symptoms of hypoglycemia during the use of desoxycorticosterone. The fact that these symptoms are not relieved by this substance, as they are by an extract from the adrenal cortex, is in keeping with the evidence that only corticosterone and related compounds have an anti-insulin effect and increase gluconeogenesis and the deposition of glycogen in the liver (92, 110, 111, 119, 121).

Resistance to infection and stress.—An extract of the adrenal cortex has been used in clinical medicine for the relief of infection and to raise the resistance against such stress as surgical treatment. At present there is no definite chemical criterion to measure its effect on patients who have intact adrenal glands but the value in clinical medicine is in some cases so apparent and the evidence gained from its use in infection is so well established that a wide trial of this type of treatment is indicated. The value of parenteral administration of extracts from the adrenal cortex, together with sodium chloride and excess fluid by mouth, has been demonstrated in a number of cases of severe infections. Its value was noted in maintenance of normal blood pressure, decrease in signs of toxicity, the avoidance of circulatory collapse, maintenance of appetite and sense of well-being, and the decrease in tendency to complications, particularly the absence of distention (122). Diphtheria toxin and antitoxin to stimulate the output of the adrenal glands are being used with promising results in the treatment of leprosy (123).

THE FUNCTION OF THE ADRENAL CORTEX

Phosphorylation.—A few years ago the suggestion was made that the adrenal cortex specifically influences phosphorylation, and a large

amount of experimental work was interpreted in terms of the hypothesis that the physiologic effect of the adrenal cortex is exerted through its influence on phosphorylation (124). This hypothesis was favorably received and widely quoted. However, the work reported during the past two years has failed in every instance to confirm the earlier observations and these experiments, together with new work, show quite clearly that the function of the adrenal cortex is not concerned primarily with phosphorylation.

The original experiments indicated that riboflavin phosphate can maintain adrenalectomized rats in good condition and that phosphorylation of riboflavin cannot proceed in the absence of the adrenal cortex. It has now been shown that neither riboflavin nor its phosphoric acid ester can replace an extract of the adrenal cortex for the maintenance of life in adrenalectomized rats. Since the effect of riboflavin and its phosphoric ester on the weight and survival of adrenalectomized rats is the same, the hormone of the adrenal cortex is not essential for the elaboration of the yellow enzyme (125, 126).

It is impossible to detect any significant difference in the cocarboxylase content of a boiled extract of livers taken from normal and adrenalectomized rats or in the ability of the livers of normal and adrenalectomized rats to phosphorylate thiamin *in vivo*. No difference can be detected in the synthesis, *in vitro,* of cocarboxylase by slices of liver taken from normal and adrenalectomized rats (127). There is no difference in the cozymase content of heart, liver, muscle, or kidney of adrenalectomized and control rats. Since this enzyme contains a prosthetic group which carries the phosphate radical the result indicates that the adrenal is not essential for the activity of this enzyme (128).

A decrease in the rate of absorption of glucose by the intestine has been interpreted as evidence for failure of phosphorylation but absorption of glucose continues at a normal rate in the toad (*Bufo arenarum*) after adrenalectomy (129), and the absorption of glucose from the intestine of rats is decreased by many factors other than phosphorylation, among them postoperative anorexia (130).

Formation and deposition of glycogen.—The increase in the excretion of sodium and the retention of potassium occur within twenty-four hours after adrenalectomy. Simultaneously with these changes, blood sugar and glycogen in the liver are decreased. From these observations it is impossible to determine which disturbance is primary and which secondary (131). A more striking and convincing answer which

shows that the hormones of the adrenal cortex are not essential is given by the observation that adrenalectomized rats which are maintained in good condition with a proper intake of sodium and chloride can deposit glycogen in the liver almost as well as does an intact animal (132).

The suggestion has been made that in adrenalectomized animals the ability to convert three-carbon substances into glucose is markedly impaired. The evidence for this conclusion was obtained by determination of the excretion of glucose in phlorhizinized, adrenalectomized rats after intraperitoneal injection of lactic and pyruvic acids and alanine (133). The conditions under which this work was carried out do not seem satisfactory to the reviewer. The substances were injected intraperitoneally dissolved in 11 cc. of water; the acids were given as sodium salts. The ability of the adrenalectomized animal to cope with this situation is known to be impaired. Any fluid injected intraperitoneally is a great strain to an adrenalectomized animal and the presence of the sodium salts of organic acids would result in accumulation of a large amount of base. These theoretical considerations render the experiment unsatisfactory, but a positive answer has already been obtained by the demonstration that casein given by mouth is converted into glucose by the phlorhizinized, adrenalectomized rat at a rate which approaches that of the phlorhizinized normal animal (95). This is a clear demonstration that the function of the adrenal cortex is not specifically concerned with the synthesis of glucose from amino acids.

The two most striking effects of the hormones of the adrenal cortex are, first, on permeability of membranes and the distribution of inorganic ions, and, second, on gluconeogenesis and deposition of glycogen in the liver. There is abundant experimental evidence that the amorphous fraction of an extract of the adrenal cortex and desoxycorticosterone have the greatest effect on the distribution and excretion of inorganic ions and that compounds with an atom of oxygen at carbon 11 affect primarily gluconeogenesis and the deposition of glycogen in the liver. In a recent review of the pituitary-adrenal relationships it was concluded that the function of the pituitary may be to preserve the lives and secretory activities of the adrenal cortical cells in the fasciculate and reticular phases of their history. The hypophysioprivic animal secretes only an immature cortical hormone. The internal layers of the cortex work over or ripen the hypothetical secretion of the outer layer (134).

It appears possible to the reviewer that this observation on the anatomic structure of the gland may have an interpretation in the chemical structure of the cortical hormones: those compounds typified by desoxycorticosterone which affect primarily the distribution of inorganic ions are elaborated by the outer layer, and those typified by corticosterone which affect primarily carbohydrate metabolism and have an additional atom of oxygen on carbon 11 may have been so "ripened" by the internal layer of the adrenal cortex.

PROGESTERONE

The Isolation and Partial Synthesis of Derivatives of Progesterone

Distinct progestational activity is manifested by 6-α-hydroxy-progesterone in the form of its acetate (135). When this substance is saponified the double bond moves from position 4,5 to 5,6 and then yields 3,6,20-pregnanetrione (136). $\Delta^{16,17}$-Pregnenedione-3,20 has been prepared from the plant steroids sarsasapogenin and tigogenin (137, 138). Hyodesoxycholic acid has been degraded to give 6-hydroxypregnane-3-ol-20-one (139) and progesterone (140).

The Assay of Progesterone

The accuracy of the method for the assay of progesterone by intrauterine application in the rabbit has been confirmed. Positive results are obtained with as little as 0.06 μg. (141). Progesterone injected with estradiol benzoate in a ratio of 1 to 50 or 1 to 100 decreases the size of the uterus and inhibits uterine estrus in ovariectomized or immature rats. The curve which represents the degree of inhibition forty-four hours after the simultaneous administration of estrone and progesterone, and the logarithm of the dose is linear. Amounts of progesterone as low as 0.08 to 0.14 mg. can be assayed on immature rats (142, 143).

The perineum of the baboon is a sensitive indicator for the female sex hormones. Deturgescence in the normal adult female baboon is a positive phenomenon due to the presence of progesterone and not only to the absence of estrone. The injection of 10 mg. of progesterone will cause a depression of the turgescent perineum which will last for from four to six days and is not succeeded by bleeding. The injection of 15 mg. of progesterone is succeeded by bleeding. By repeated in-

jections of progesterone in the deturgescent or first part of the cycle
the cycle may be prolonged indefinitely, or by early withdrawal the
cycle may be shortened or confined to normal length. The time of
onset of bleeding after withdrawal of progesterone depends on the
quantity used (144).

The uterus and perineum of the baboon, after they have been sen-
sitized with estrone, have a greater affinity for progesterone than for
estrone, and progesterone is active in the presence of large quantities
of estrone. Deturgescence of the perineum without bleeding makes
possible an easy and accurate assay of progesterone uncomplicated by
operative procedures (145).

During a complete menstrual cycle the progesterone in the blood
of normal *Macaca rhesus* varies between a maximum of 0.25 to 2.5 µg.
and a minimum of 0.06 to 0.12 µg. per cc. of blood (146).

The Metabolism of Progesterone

Progesterone is not excreted in biologically active form by young
women after three intramuscular injections of 20 mg. each (147).
Further studies in the functional output of the corpus luteum through
the recovery of progesterone as pregnanediol glucuronide have been
made (148). The conversion of progesterone to pregnanediol is im-
mediate and maximal when all the normal mechanisms are operative
(the luteal phase of the menstrual cycle—early pregnancy). Under
these conditions there is no difference between the recovery of in-
jected progesterone and injected sodium pregnanediol glucuronidate.
The recovery of injected progesterone as pregnanediol glucuronide is
poor under some conditions (hysterectomy, atrophic endometrium,
failure of conjugation), but the presence of normal proliferative endo-
metrium is not essential for the conversion of progesterone into preg-
nanediol (149). This is also shown by the recovery of pregnanediol
glucuronide from the urine of men after the injection of progesterone
(150). Pregnanediol glucuronide may be recovered with a yield from
0 to 50 per cent when it is given by mouth to healthy men (151).

Although progesterone is reduced to pregnanediol and excreted as
its glucuronide in man, the injection of progesterone into rabbits, cats,
and adult monkeys is not followed by the excretion of pregnanediol
glucuronide in any case and after hydrolysis no free pregnanediol is
in the urine of a monkey after the injection of 80 mg. of progesterone
(152). The urine of a pregnant monkey does not contain pregnanediol

and the injection of more than 1 gm. of progesterone during twenty days into a pregnant monkey fails to produce pregnanediol in the urine (153).

Pregnanediol glucuronide may be hydrolyzed by an enzyme which is formed by a microorganism present in the urine of all female patients. It is necessary therefore to determine free pregnanediol in addition to the conjugated form. Determination of the amount which is present in the free form may not be a quantitative index of the total amount excreted in the conjugated form since enzymic hydrolysis of the conjugated form may not be complete because of overgrowth of bacteria which kill the organism that elaborates the enzyme (154).

Desoxycorticosterone exerts a progestational effect in spayed immature rabbits and in spayed adrenalectomized cats pretreated with estradiol benzoate. The uterine response in the adrenalectomized cats is comparable to that obtained with progesterone (155). This observation suggests that the high order of progestational activity may be due to a chemical change in the structure of desoxycorticosterone which results in the formation of progesterone by the reduction of the hydroxyl group on C_{21}. Such a reaction does occur in healthy men; desoxycorticosterone is converted into pregnanediol glucuronide (86).

In immature castrated rats, androgenic effects follow the subcutaneous administration of 2 mg. of progesterone daily for a period of ten days, although the same dose has only a slight androgenic effect if given intraperitoneally. The negative results reported in the literature for the androgenic effect of progesterone may be due to inadequate total dosage or to administration by the intraperitoneal route (156), but it seems possible that the positive evidence for androgenic activity may be due to the degradation of progesterone into compounds which have adrogenic properties.

The Influence of Progesterone on the Sex Organs and Mammary Glands

Large amounts of progesterone administered to the adult rat and mouse cause inhibition of follicular maturation (157, 158), involution of the corpus luteum, and marked atrophy of the ovary, but in pregnant mice progesterone does not produce significant atrophy of the ovary or any detectable change in the corpora lutea of gestation. Delivery, and onset of lactation, are not prevented by massive doses of progesterone. This finding is not in accordance with the assumption

that discontinuance of the production of progesterone is the cause of delivery at term and of the initiation of the secretion of milk (159). The daily injection of 5 mg. of progesterone for five to six days prevented secretion of milk after delivery in six patients and reduced it to a duration of two days in three patients (160). In spayed rats progesterone produces progestational changes in the endometrium, vaginal mucification, and development of the mammary glands similar to that seen in late pregnancy. When used in large amounts progesterone can exert all of its characteristic action in spayed female rats without sensitization with estrogens (161).

Although progesterone causes lengthening of the ovipositor of the female bitterling in greater dilution than does testosterone propionate this effect is relatively slight when compared with that of the male hormone. The maximum response to progesterone takes place during the first six to eight hours, that to testosterone only after twenty-four to seventy-two hours (162).

Anhydrohydroxyprogesterone.—For the production of progestational proliferation, anhydrohydroxyprogesterone has about a tenth the potency of injected progesterone, but it produces growth of the uterus of the spayed rat without previous treatment with estrogen. Its effects on the uterus are very similar to those produced by progesterone following administration of estrone. In addition to the high activity when given orally, anhydrohydroxyprogesterone seems to be the only compound so far described which has progesterone-like, metrotrophic, androgenic, and estrogenic properties (163, 164). Unlike testosterone it does not stimulate the hypophysis to release gonadotropic hormone (164). When given by mouth to a young woman no biologically active progesterone and no sodium pregnanediol glucuronidate appeared in the urine. No alterations occurred in the titers of androgens in the urine during the injection of this steroid derivative (165, 166).

ESTROGENS

The Synthetic Preparation and Isolation of Estrogens

The most important contribution concerned with the synthetic preparation of estrogens is the total synthesis of equilenin. The total synthesis of this sex hormone places the preparation of at least some of the estrogens on a basis independent of the steroid nucleus ob-

tained from natural sources. The reduction of equilenin to estrone and estradiol has not yet been carried out with a satisfactory yield but in all probability this step will be achieved (167). Of the four optically active stereoisomers only the d-equilenin which is the natural hormone possesses appreciable estrogenic activity. Desoxyequilenin obtained by removal of the hydroxyl group at carbon 3 is without estrogenic activity (168). Δ^6-Isoequilin, prepared from 7-hydroxyestrone, possesses about a third the physiologic potency of estrone (169). The introduction of a ketone group in position 6 of α-estradiol decreases the estrogenic activity to a fourth that of α-estradiol (170). Estradiol has been isolated from human pregnancy urine (171, 172) and from the nonketonic fraction of the human placenta (173). A ketone characterized as $\Delta^{5,7,9}$-estratrienol-3-one-17 has been isolated from the urine of pregnant mares. This is a neutral isomer of estrone in which ring B instead of the ring A is benzenoid (174). The estrogenic hormones in human follicular fluid are present in the combined as well as in the free form (175). The concentration of estrogen per unit volume of follicular fluid in the mare is constant throughout the cycle but the total estrogen is a third as great in the nonestrous phase as during estrus (176). The yolks of freshly laid eggs contain 5 rat units of estrogenic material per kilogram of yolk (177). Estrone has been isolated from ox adrenal glands (178). Glucosidic derivatives of estrogens have been prepared through treatment of estrone with acetobromoglucose. The glucoside derivatives of estrone and estradiol were both more active than the corresponding free estrogens (179).

The Colorimetric Determination and Assay

Estrone, because of its phenolic group, may be determined colorimetrically through a coupling reaction with diazotized dianisidine (180), or with guaiacol sulfonic acid which gives a reaction specific for this keto estrogen (181). The sensitivity for the biologic assay for estrogenic compounds may be increased several hundred times, depending on the amount used, by intravaginal application of a glycerin solution containing the estrogenic substance instead of by subcutaneous injection (182).

The Metabolism and Stability of Estrogens

Estrone, separated in crystalline form and identified as the benzoate, was excreted by the guinea pig after the administration of estra-

diol even in the absence of the ovaries (183). Estradiol is inactivated by liver and kidney *in vitro* but not by other tissues. The inactivation is probably not through conjugation or conversion to a less active form but is due to destruction by an oxidative enzyme. Estrone is increased in potency by incubation with minced uterine and other tissues, probably because of the conversion to estradiol. The liver also apparently first converts estrone to the diol form which in turn is inactivated. The better results which are obtained with estriol administered orally are probably due to its greater stability: it is only mildly affected by liver tissue in contrast to the great destruction of estrone and estradiol (184).

The production of gynecomastia in advanced cirrhosis of the liver may be explained through the failure of the cirrhotic liver to inactivate estrogens which are present in the free but not in the combined form (185). Damage of the liver produced by carbon tetrachloride increases the effectiveness of administered estrogens, presumably by interfering with detoxication of the compounds by the liver (186). Estrone is readily inactivated by hydrogen peroxide in an alkaline solution or in the presence of catechol oxidase. Tyrosinase also inactivates estrone and estradiol (187).

CHANGES INDUCED IN THE UTERUS BY ESTRIN

The injection of 0.1 μg. of estradiol in an immature rat produces hyperemia of the uterus which closely parallels the early increase in weight and the content of water. This effect does not appear to be mediated through acetylcholine but there is evidence for the presence of a histamine-like substance (188).

The effects of estrin on the uterus may be divided into two groups: (*a*) those specific actions, proliferation and hypertrophy of the cells of the lining epithelium and the hypertrophy of the endometrial glands, which do not depend on hyperemia; and (*b*) those which are secondary to the hyperemia (189). The high level for the increase in the water content of uterus produced by an estrogen is reached in six hours and is followed by a loss of water in twelve hours (190). Progesterone and large amounts of androgens inhibit this effect (191). Although estrone does not affect the rate of anaerobic glycolysis of isolated uterus *in vitro,* six to twelve hours after the injection of estrone into rats the rate of anaerobic glycolysis of the isolated uterus is increased. The maximal increase is not reached until twenty-four

to thirty-six hours, but continued injections of estrone do not maintain the high rate of glycolysis. Thirty-six hours after the injection of estrone the consumption of oxygen is increased although not as much as is the rate of anaerobic glycolysis (192). The uptake of oxygen by the uterus appears to be dependent on glycolytic and aerobic mechanisms (193).

Associated with the increased rate of physiologic processes in the immature rat uterus after a single dose of estrogen there are changes in the concentration of inorganic ions. During the first six hours after the administration of estrogen there is an increase in water and chiefly extracellular electrolytes and in the ratio of potassium to phosphorus. During the following twenty-four hours there is a rapid growth of new protoplasm with a gradual return of the concentration of electrolytes to normal. The increase in the extracellular phase is from 50 to 75 per cent while that in the cells is only a third as much. At first the cells take up a normal quantity of potassium but definitely less than their quota of phosphorus and probably of solids. This is followed by a marked growth during which the cells take up approximately normal amounts of cell constituents. The changes observed in the first six hours are of interest because they probably precede mitotic activity (194). The changes in the genital organs during the estrous cycle and after the injection of gonadotropic hormones which have been extensively studied in the rat, mouse, and dog have been further investigated in the dog (195, 196, 197). In this connection vaginal biopsy is recommended. Because of its innocuousness it can be repeated at intervals of a few days and the slow evolution of changes in the epithelium can be shown in serial form. This procedure is preferable because of its exactitude in comparison to the vaginal smear or washings (198).

INFLUENCE OF THE ADRENAL CORTEX ON ESTRUS

Adrenalectomy sensitizes the vagina to estradiol benzoates and produces continuous estrus in adult ovariectomized rats treated with estradiol benzoate. Desoxycorticosterone inhibits this effect and suppresses the estrous response (199, 200).

THE EFFECT OF ESTROGENS ON GROWTH OF SEX ORGANS

The formation of the corpus luteum can be prevented by the injection of estradiol benzoate but if the injection is made after the

luteal phase has started the output of pregnanediol is decreased by estradiol benzoate. The formation of corpus luteum is not prolonged by the administration of estrogen (201). Estrone and estradiol move the sex ratio in the chick embryo in the female direction. Small amounts of estradiol benzoate cause persistence of the left Müllerian duct and large amounts cause persistence of the right Müllerian duct of both sexes (202). Chick embryos treated with estradiol benzoate retain signs of strong feminization during early life of the birds. Two months after hatching 50 per cent are normal females and the other 50 per cent possess gonads which contain in every instance testicular tissue. In the females the Müllerian ducts are entirely normal but in the male they are enormously swollen. The sex ratio of the birds killed nine months after hatching is normal. The feminized males cannot maintain their female sex organs during postnatal life and the ovaries produced by the estrogen are transformed into testes; the ovarian remnants persist in only a few cases (203). Estrone and estradiol propionate produce the reversal of sex in male salamander larvae, *Amblystoma maculatum* and *A. tigrinum,* but testosterone does not exert a corresponding effect upon genetic females (204). The injection of estrone into salamander larvae, *A. tigrinum,* at the beginning of sexual differentiation produces atypical gonads which are ovo-testes so modified as to resemble retarded ovaries with irregular medullary remains (205).

The Influence of Estrogens on Bones

Estradiol benzoate in immature female and male mice stimulates proliferation of new bone, especially in the medullary cavities of the lower end of the femur and upper end of the tibia, associated with sclerotic changes and inhibition of growth. The pubic bones when involved are more or less resorbed, depending on the dosage of estrogen and the extent of the experiment (206). Continued administration of large doses of estradiol benzoate in mice produces significantly higher concentrations of inorganic constituents in the femurs and pelves. The higher content of each of the untreated females as compared with untreated males indicates the influence of the animals' own hormones on skeletal growth. Testosterone does not increase the concentrations of inorganic constituents in the bones and it prevents estradiol from exerting its influence when the two are given together (207).

The Influence of Estrogens on the Production of Tumors

A pellet of estradiol implanted beneath the skin may produce uterine and extrauterine tumors in guinea pigs. The degree of tumorogenesis is much greater when the estrogen is administered in the form of tablets than when injected (208). In twenty-eight of forty-nine female rats, tumors developed in the mammary glands after treatment with tablets of estrone implanted in the subcutaneous tissue. In the tumors there was extreme cellular hyperplasia but no evidence of invasion of the stroma or rupture of the basement membrane by tumor cells (209). The transplantability and rate of growth of mammary fibroma are markedly enhanced by the presence of physiologic levels of estrogens in the host rats. The tumor-stimulating effect of estrogens was greater in castrated male rats than in castrated female rats. With the onset of malignancy the connective tissue tumors lose their responsiveness to the influence of hormones (210).

Women with cancer appear to be unable to bring about the conversion of estrone to estriol to any appreciable extent even when progesterone is injected. This is true although the total output of estrogen before injection may be higher than in noncancerous women (211).

The Effect of Estrogens on the Blood Calcium of Fowls

At the time of calcification of eggs in fowls there is a marked rise in the calcium content of the blood but there is not sufficient evidence to associate this rise with estrogenic hormones. Thousands of times the calculated amount of estrogens contained in the fowls would be required to raise the blood calcium to the observed levels (212) although when massive doses of estrone are injected there is a marked rise in the concentration of calcium in the blood (213).

The Influence of Estrogens on the Mammary Gland and Other Effects

Estrone in oil rubbed into the skin of the rudimentary mammary glands of young male rabbits causes growth of those glands but there is no growth of control glands treated with oil only (214). Estrone applied to the nipple area of an immature male monkey resulted in distinct development of this gland but not of the untreated one. This

observation is evidence for the direct action of estrone and does not support the view that estrogens stimulate mammary growth through mediation of the pituitary gland (215).

Adaptation of growing rats to the continued injection of estradiol is possible although antihormones are not formed. Long-continued administration causes loss of weight and eventually death (216). There was a high incidence of urinary calculi (33 per cent) in 151 mice that were five months old or older after from nine to twenty weekly injections of estradiol benzoate (217).

An estrogenic hormone (amniotin) has been used for local application in the treatment of laryngeal papillomata. This is of particular advantage in cases in which operation is not feasible because of the probability of return of the condition (218).

Reviews of the chemical nature of the steroids of the sex and adrenal glands (219) and of the clinical application of the sex hormones in conditions of gonadal hypofunction and diseases of the ductless glands (220, 221) summarize our present knowledge.

Diethylstilbestrol and Related Compounds

From anethol 4:4'-dihydroxy-γ,δ-diphenyl-n-hexane (hexoestrol) has been prepared and found to be somewhat more active than diethylstilbestrol when injected into ovariectomized rats (222, 223, 224, 225). Diethylstilbestrol can be determined in quantities as low as 1 µg. per cc. by a color reaction given with antimony pentachloride (226). The close parallelism shown between diethylstilbestrol and estrone has been further studied in its effects on the atrophy of the gonads and reduction or suppression in body growth associated with an increased weight of the adrenals and pituitary. Of the estrogens, the stilbene derivatives are the only ones that produce toxic degeneration of the liver and kidney (227, 228, 229, 230). Diethylstilbestrol, even in large amounts, does not exhibit progestational activity on immature female rabbits. The inhibition of body growth is produced by smaller amounts than those required to atrophy the gonads and the effect on the growth of bones is brought about by as little as 10 µg. Cessation of treatment with diethylstilbestrol is not followed by reappearance of the growth of bones, although after treatment with estrone is stopped there is recurrence of bone growth. A high percentage of the rats die within three weeks with a daily dose of 200 µg. of diethylstilbestrol (231, 232). Although lactation may be reduced by a daily

dose of 2 or 3 μg. (231) the application for thirty days of diethyl-stilbestrol dipropionate in ointment to the udder of a virgin female goat produces copious secretion of normal milk. All attempts to induce lactation in male goats failed even when progesterone was also given (233). Diethylstilbestrol increases the concentration of total fatty acids, phospholipids, and cholesterol in blood more strikingly than does estrone or estradiol (234).

The application of the experimental findings with the synthetic estrogens, particularly diethylstilbestrol, to clinical medicine with the report of striking results in demasculinization and feminization (235) has been reviewed (236). The use of diethylstilbestrol and its esters for the relief of the syndrome of artificial and physiologic menopause (237) and for the therapeutic qualifications of the natural estrogens has shown the efficiency of the synthetic preparation. However, extreme caution with its use in clinical medicine is advised until after more intensive study of its toxicity (238). Its use in some cases is limited because of the persistent nausea and occasional vomiting (239). A review of the clinical use of diethylstilbestrol indicates that the natural estrogens may be eliminated in clinical practice, particularly if the toxic side effects can be reduced by modifications of the chemical structure (240). In this respect the results with hexoestrol are encouraging since only about 5 per cent of the cases treated with hexoestrol show toxic effects compared with 22 per cent of those treated with diethylstilbestrol (241). Diethylstilbestrol dipropionate has been used successfully by the implantation of 100 mg. hard pellets in six women. The average daily absorption was between 0.125 and 0.250 mg. The pellets became encased in a fibrous capsule but the amount absorbed was only a fraction of that required for oral administration or injection (242).

ANDROGENS

Preparation, Extraction, and Colorimetric Determination of Androgens

Androstadien-ol-17-one-3, an androgen with two double bonds in the A ring (243), and a methylated androgen 6-methyl-Δ^4-androstene-3,17-dione have been prepared. The latter has an activity about equal to androstenedione (244). Extensive investigations concerning the

determination of keto steroids have shown the importance of details in methods employed for hydrolysis, extraction, purification, and production of color. Modifications which increase the accuracy of the determination are hydrolysis with steam, continuous extraction with carbon tetrachloride, a small volume of urine and solvent, and the elimination of the use of ether (245). Other modifications are the adsorption of the steroid fraction from a solution in benzene on magnesium oxide, followed by elution with ether. The androgens may be further fractionated into α- and β-steroids with digitonin. In most cases the percentage of the β-form is small but in a case of virilism 40 per cent of the total androgens was found in the β-fraction (246). Purification of the androgens with the Girard reagent dispenses with decolorization with charcoal (247, 248).

Because of the subjective errors inherent in visual colorimetry the use of the photoelectric instrument is preferable. For clinical purposes the Oesting colorimeter is adequate but detailed investigation of the color produced with pure androgens compared with the color given by extracts of urine emphasizes the difficulties in the quantitative determination of androgens in a mixture (249 to 253). A comparison of the colors given by the Zimmermann reaction with dinitrobenzene on forty-one pure compounds indicates that the colorimetric method is of considerable value for the determination of pure androsterone and estrone. However, the results by the colorimetric method are always considerably higher than the values obtained by assay on the capon (254).

Bio-assay of Androgens

Comparison of the Hellige colorimetric determinations with bio-assay based on the response of the epithelium of the seminal vesicles of immature castrated rats indicates that both methods are satisfactory as clinical guides for treatment (253). For the assay of small amounts of androgen the weight measurement of the ventral prostate is the most sensitive indicator. For amounts of androgen greater than normal the weight of the empty vesicles appears to be the best measurement because of slightly greater reliability (255). The stimulating effect of androgens on the seminal vesicles of the castrate rat is more sharply defined by the use of colchicine (256, 257). The variables which affect the estimation of androgenic activity are described in a comprehensive review (258).

Metabolism and Stability of Androgens

Androsterone was isolated from the urine of men after the oral administration of androsterone, testosterone, etioallocholane-3(α)-17-diol, etiocholane-3,17-dione, and etiocholene-(4,5)-3,17-dione (259). In man, even with evidence of deficient testicular secretion, testosterone is converted into androsterone. Etioallocholane-3,17-dione may be an intermediate during the conversion since androsterone is excreted after the administration of this substance to man (260). Testosterone propionate is converted into etioallocholanol-3,β,17-one in adult guinea pigs (261). That the liver may destroy androgens is indicated by implantation of testicular tissue in immature rats so that the venous drainage passed through the liver in one but not in another group of animals. In the first group there was lack of androgenic activity, but some androgenic effect was observed in the second group (262). The increased effectiveness of methyl testosterone over that of testosterone propionate when given orally to men and rats indicates different routes of absorption from the intestinal tract rather than different sites of inactivation, since both androgens are destroyed in the liver. It is probable that methyl testosterone is absorbed principally from the lacteals and lymphatics (263).

Androgenic Activity of Desoxycorticosterone

When tested on the capon, mouse, and rat the androgenic activity of desoxycorticosterone is approximately a thirtieth as great as that of androsterone (264). Desoxycorticosterone has no effect on the chick comb (265). It inhibits the development of the immature rat ovary and in the adult rat it produces atrophy and cystic atresia of the follicles in the ovary (158); however, after castration it does not prevent regression of the ventral prostate in the male rat (266). In a daily dose of 10 mg. desoxycorticosterone is an effective stimulator of the secondary sex organs of the mature castrate female monkey (267).

The Influence of Testosterone on the Uterus

Testosterone propionate exerts both an estrogenic and progestational effect on the uterus of ovariectomized rats resulting in marked growth of the organ and a pregnancy-like state of the endometrium. The estrogenic effect appears early and is progressive throughout the

duration of the treatment. The progesterone effect does not appear before the eighth day and is not complete before the fifteenth. Longer treatment maintains the progesterone appearance of the endometrium but produces cystic changes (268).

In the monkey a progestational endometrium is not attained with testosterone alone or in combination with estrogens. Unlike the rabbit uterus, that of the monkey does not respond to testosterone as it does to much smaller doses of progesterone. Testosterone does not exert an antagonistic action either to estrogens or to progesterone when injected concomitantly with these hormones. Although testosterone does not produce an estrogenic action in the uterus of the monkey it is able to maintain an endometrium already built by estrogen (269). Testosterone, testosterone propionate, and androstenedione will maintain pregnancy with resulting living fetuses in rats castrated during the latter half of pregnancy (270). Testosterone propionate given subcutaneously with estradiol benzoate in a ratio of 1 to 250 could not completely neutralize the effect of the estrogen. The vaginal epithelium was built up and there was an increase in size of the uterus but the scaling off of the cornified cells in the vagina did not take place (271).

EFFECT OF ANDROGENS AND ESTROGENS ON SEX ORGANS

Testosterone propionate suppresses the weight of the ovaries in young female chicks (272) and rats (273, 274) and transforms ovaries into testes in frog larvae (275). The inhibition of the development of the sex organs caused by injection of testosterone at an early age in the rat persists and prevents the onset of puberty (273). Testosterone propionate caused sex reversal in 50 per cent of female fish (*Xiphophorus helleri*) (276).

The influence of testosterone during early life which directs the development of undifferentiated embryonic organs into the male type can also transform the fully differentiated postnatal female genitals and type of hair of the rat in the male direction (277). The injection of 1500 μg. of androsterone or dehydroandrosterone prevented the Müllerian ducts from originating in nearly all embryos, and this effect was not counteracted when 500 μg. of estradiol benzoate was injected simultaneously (202). The injection of estrone in young male rats prevents the development of the testes, probably through inhibition of the gonadotropic action of the hypophysis. When testosterone is

injected simultaneously the effect of estrone is nullified. Testosterone propionate has a direct gonadotropic effect on the testis independent of the hypophysis in young male rats (278) and in mice the simultaneous administration of testosterone with estrone prevented metaplasia in the anterior part of the prostate although it did not completely suppress other pathologic changes (279).

The antagonistic action between androgens and estrogens has been demonstrated by their simultaneous administration to pregnant rats: the usual masculinizing effects of androgens and feminizing effects of estrogens on the offspring are prevented by the presence of the opposite hormone (280). Although testosterone does not have a stimulating action of itself, it nullifies, counteracts, or neutralizes the usual action of estrin on the human vagina (281). *Trans*-androstenedione is estrogenic in female and androgenic in male rats (282). Estrogens do not stimulate secondary sexual characteristics in either sex of the black-crowned night heron or the starling. The changes observed in these birds are apparently produced by an androgen in both sexes (283, 284).

The effect of androgens on the contraction of the uterus, tubes, and ligaments.—Testosterone propionate reduces the force of the tubal contractions of the oviducts of rabbits. There is a marked inhibitory effect on the tubal muscle which diminishes the general tonicity and amplitude of the contractions (285). In the mouse testosterone propionate interrupts pregnancy and in adequate doses may cause the retention of blastocytes in the oviducts (286). Progesterone, in the absence of estrogenic hormone, has little effect on the human myometrium but testosterone alone produces contractions which resemble those seen after simultaneous administration of estradiol and progesterone (287). Testosterone propionate injected subcutaneously in large amounts inhibits the interpubic ligaments and pelvic changes during pregnancy (288).

EFFECTS OF ANDROGENS ON VARIOUS ORGANS

There are no acute effects from the injection of sex hormones (289). However, long-continued administration to the Little dba strain of rats showed that animals of each sex are better able to tolerate the sex hormone of the opposite sex. Testosterone propionate is not fatal, but causes a decrease in gain in body weight in the males. Estradiol propionate is frequently fatal, more in females than in males.

Testosterone when given in large amounts nullifies in part but not completely the fatal toxic properties of estrogen (290). Testosterone, methyl testosterone, androstenedione, and androstanediol are about equally effective in inhibiting the response of spayed mice to estrone, but androsterone, transdehydroandrosterone, and androstanedione are ineffective in doses up to 2 mg. (291). Testosterone produces an increase in compensatory hypertrophy after unilateral nephrectomy (292) and when given over a period of two to three weeks causes marked enlargement of the kidneys of the mouse, with pronounced hypertrophy of the epithelium of proximal and distal convoluted tubules and of the epithelium lining the parietal lamina of Bowman's capsule (293). Besides causing an enlargement of the kidney testosterone protects the tubular cells against the toxic effects of mercury chloride (294).

The Influence of Testosterone on the Thymus

Castration brings about hypertrophy of the thymus in rats, but the injection of testosterone propionate is followed by atrophy in both normal and castrated rats of either sex (295, 296).

The Influence of Testosterone on Bones

Treatment of a fifteen-year-old eunuch with testosterone for two years increased the rate of growth but did not affect the closure of the epiphysis, as evidenced roentgenographically. Marked stimulation of growth did not begin until the dose of 30 mg. weekly had been maintained for several months (297). However, the warning is given that testosterone propionate increases the rate of epiphyseal maturity to such an extent that dwarfing might be caused if large doses were administered to patients who had not obtained their normal adult structure (298).

The Effect of Androgens on Physiologic Processes

Testosterone propionate increases the glycogen of the muscle of normal male rabbits slightly but does not change the content of phosphagen or adenyl pyrophosphate. Castration produces a decrease in the content of glycogen of the muscle but the normal content is restored by treatment with testosterone propionate. The glycogen in heart muscle decreases for some weeks after castration but is again normal at the end of thirty-one weeks (299). The administration of tes-

tosterone propionate to a eunuchoid decreased the excretion of so-
dium, potassium, and chloride and caused a gain in weight. The nitro-
gen stored was retained in great part for weeks. All the changes indi-
cate a somatotropic influence of androgens and furnish a clew to the
explanation of the unusual body growth concomitant with precocious
puberty (300).

*The influence of the androgens on metabolism of creatine and
creatinine.*—Creatinuria does not develop in castrated adult male rats
but testosterone propionate causes a decrease in exogenous creatinuria
which is greater than in the normal animal. The administration of
testosterone propionate and creatine is followed by changes in crea-
tinuria which parallel changes in the body weight until the body weight
reaches a high level, at which time intense creatinuria reappears even
though administration of androgen is continued with the ingestion of
creatine. Creatinuria falls to an insignificant value after the adminis-
tration of creatine and androgen is discontinued. The muscle tissue
of castrated rats appears normal under these conditions (301). Young
male monkeys usually excrete creatine and show an impaired retention
of exogenous creatine. Testosterone propionate causes an abolition of
creatinuria and increases the capacity to retain creatine. Castration
of mature monkeys produces a condition similar to that in castrated
rats. Estradiol benzoate is without effect on the excretion of creatine,
and excretion of creatinine was not affected by any of the procedures
(302).

The Androgens in Clinical Medicine

The influence of testosterone on lactation.—Testosterone propio-
nate inhibits lactation post partum: in only 2 per cent of 108 cases
was there failure and all patients obtained relief from engorgement or
distention and pain in the breasts (303).

The ratio of androgens to estrogens.—The administration of estro-
gens either orally or by injection will reduce elevated amounts of
androgen in cases in which the ratio of androgen to estrogen is not
normal. In no instance were the amounts of androgen reduced lower
than the average normal levels by the administration of estrogen
(304). There may be a significant decrease in the excretion of estro-
gens by women with acne vulgaris without a decrease in the excretion
of androgens. A change in the ratio of estrogen to androgen may be
an etiologic factor (305). These observations are in keeping with
the suggestion that a permanent condition of chemical hermaphro-

ditism persists throughout life in men and women. Disturbances in the ratio of maleness to femaleness may give rise to clinical manifestations (306). The administration of testosterone propionate to human castrates increases the excretion of estrogen and produces a slight rise in the excretion of androgen (307).

Individuals with carcinoma tend to excrete less androgen than noncancerous persons. After the administration of estrogen the urinary output of androgen increases but to a lesser extent in patients with cancer. The simultaneous administration of estrone and progesterone increases the output of androgen slightly (308).

The excretion of androgens by normal men has been compared with the excretion by men with endocrine syndromes relating particularly to the pituitary gland (309).

Androgens and the adrenal cortex.—Determination of the androgens in the urine of eunuchs, eunuchoids, ovariectomized women, patients with Addison's disease, and a patient with deficiency of the pituitary, indicates that androgens and 17-ketosteroids may be derived from both the gonads and the adrenal cortex. The determination of the excretion of 17-ketosteroids has diagnostic value in cases of adrenal cortical tumor, and has confirmatory value in cases in which a lowered function of the adrenal cortex is suspected (310, 311). Examination of the androgens excreted by a eunuch indicates that *trans*-dehydroandrosterone is derived from the adrenal cortex which may be more active in a eunuch than in a normal subject. Androsterone and etiocholane-3-α-ol-17-one were also isolated (312).

THE ANTERIOR PITUITARY GLAND

Separation and Investigations of the Chemical Properties of the Gonadotropic Hormones of the Pituitary, Serum, and Urine

Definite and substantial progress in the difficult problems concerned with the fractionation of the hormones of the anterior pituitary is indicated by the separation by three different groups of investigators of that fraction of the protein hormones which stimulates the interstitial cells (luteinizing hormone) of the gonads (313 to 318). This protein contains glucosamine and a hexose which is probably mannose. These constituents appear to be present in an approximately 1:1 ratio (313). The follicle-stimulating hormone has been separated as a quite separate fraction (316, 317, 318).

The gonadotropic hormone in pregnant mare serum has now been purified so that 1 mg. contains between 4,000 and 7,000 rat units (319). Investigations of the biologic activity of this highly purified material show that the response cannot be distinguished from that of untreated serum, indicating the presence of a single hormone in the serum (320).

A cataphoretic study of the gonadotropic substance separated from pregnancy urine indicates that this protein hormone is very nearly electrophoretically homogeneous and in the ultracentrifuge it behaves as a single component. The molecular weight is between 60,000 and 80,000 (321). Chemical studies indicate that it is a glycoprotein and that the carbohydrate present is galactose. The carbohydrate portion of the molecule appears to be built up of hexosamine digalactose units (322).

PURIFICATION OF OTHER HORMONES OF THE ANTERIOR PITUITARY

The gradual addition of ammonium sulfate to an extract of the anterior pituitary with careful control of the pH brings about successive fractionations and separation of the hormones (317, 318, 323, 324). Further purification of the thyrotropic (325) and the adrenotropic (326) fractions indicates progress with the preparation of these hormones. The homogeneity and mobility of the highly purified lactogenic hormone have been studied through its electrophoretic behavior (327). From the ultraviolet absorption spectrum the presence of tyrosine and phenylalanine is indicated (328).

STABILITY OF THE HORMONES

Treatment of an extract of the anterior pituitary with cysteine leads to a purification which increases the growth-promoting activity and markedly decreases the concentration of the thyrotropic, lactogenic, and gonadotropic hormones. The growth-promoting effect cannot be attributed to any of the "target organ" hormones and apparently is not due to a synergistic action of the other endocrine glands (329, 330). Acetylation of the gonadotropic hormones with ketene occurs in successive steps, first with the amino groups and finally with the phenolic groups. The physiologic activity of the gonadotropic hormones of the pituitary and of pregnant mare serum is dependent on the free amino groups. The physiologic activity of the gonadotropic principle in the urine of pregnant women is destroyed only after

acetylation of the phenolic groups (331). The potency of all gonadotropins thus far investigated is destroyed by treatment with thiol compounds (332). The activity of prolactin is destroyed with phenylisocyanate which reacts with the free amino groups (333).

The follicle-stimulating hormone is not inactivated by picric or flavianic acid (334), but it is apparently less stable to heat than the interstitial cell-stimulating (luteinizing) fraction (335). Proteolytic enzymes destroy the interstitial cell-stimulating activity more easily than the follicle-stimulating hormone (336, 337, 338, 339). Previous treatment with trypsin is recommended for the destruction of luteinizing, lactogenic, and thyrotropic hormones. From the solution a fraction rich in follicle-stimulating hormone can be separated. This product contains carbohydrate and is inactivated by diastatic enzymes and cysteine (340). Incubation of an aqueous suspension of ground pituitary glands permits the proteolytic enzymes which are present to act on the luteinizing hormone with destruction of its activity. The follicle-stimulating principle which remains in the solution may be further purified (341).

Biologic Effects of Hormones of the Pituitary

The resistance of mice to insulin after the injection of various preparations of the hormones from the anterior pituitary gland indicates that the glycotropic effect is produced by the adrenotropic factor. This hormone from the pituitary, as well as extracts from the adrenal cortex and crystalline corticosterone acetate, produces definite glycotropic effects, indicated by increased resistance to insulin convulsions (342).

The response of the ovary and uterus is augmented if the gonadotropic hormone is first adsorbed on zinc hydroxide. If the hormone and zinc salt are injected separately no augmentation is observed. The preparations from pregnancy or menopause urine, pregnant mare serum, and normal male urine are not augmented by the addition of zinc or copper salts (343). The manner in which the hormones are adsorbed on the zinc is of highest importance (344). Chlorophyll produces a slight augmentation with the gonadotropic hormone of the pituitary, an extract of male urine is not affected, and the hormone from mare serum is completely inhibited (345).

The many factors which influence the biologic behavior of the purified gonadotropic fractions from the pituitary, in respect to the phenomena of synergism, luteinization, and antagonism, are still

under intensive investigation and are a subject of dispute (343, 344, 346). One important variable is the strain of rats used for the biological tests (347).

A method for the assay of the follicle-stimulating hormone is furnished by the cornification of the vaginal epithelium of immature female rats; the presence of the luteinizing hormone has little influence on the reaction. For the assay of the luteinizing hormone a strictly specific test is furnished by activation of the melanophores and feather germs of African weaver finches. The appearance of a black dot or bar on the otherwise white abdominal feathers indicates a positive reaction (348). By the use of these criteria the amounts of follicle-stimulating and luteinizing hormones in human pituitary glands have been shown to vary over a wide range with age and with pregnancy. The human gonadotropic complex is usually rich in the follicle-stimulating hormone while the luteinizing hormone is present only in traces (349). For the assay of unfractionated pituitary extract the mouse uterus is about sixty-six times as sensitive as the rat ovary and about ten times as sensitive as the chick testes. In the assay of the preparation from pregnant mare serum the mouse uterus is about sixty times as sensitive as the rat ovary and ninety times as sensitive as the chick testes. For the assay of the preparation from normal male urine the mouse uterus is about ninety times as sensitive as the rat ovary and about fifty-five times as sensitive as the rat uterus. The response of the chick testes to normal male and menopause urine is doubtful. The mouse uterus is about thirty times as sensitive as the rat ovary to the gonadotropic hormone of menopause urine (350).

Examination of royal jelly of the honeybee has failed to indicate the presence of any principle which can cause follicular stimulation in the immature white rat (351).

The metabolism of the thyrotropic and gonadotropic hormones.— The metabolism of the thyrotropic and gonadotropic hormones has been investigated with significant results. Examination of the urine of normal and thyroidectomized guinea pigs before and after the injection of the thyrotropic hormone indicates that the hormone is excreted in the urine only in the absence of the thyroid gland. The same result was obtained with the thyroidectomized rabbit. The urine of castrate male and female guinea pigs but not that of intact animals injected with the gonadotropic hormone from the pituitary produced the typical effects of this hormone.

The thyrotropic hormone was also partially removed from solution

when introduced into a saline suspension of thyroid tissue. Muscle tissue apparently possessed less activity in this respect. Gonadotropic hormone was partly removed from solution when added to a saline suspension of ovarian tissue. A suspension of muscle or liver tissue had less activity, if any. These experiments suggest that the thyroid gland and gonads play a considerable part in the removal of the thyrotropic and gonadotropic hormones from the blood (352). A recent review of the chemical nature of the hormones with a summary of the physiologic effects of each fraction is available (353).

The anterior pituitary gland and the pancreas.—The administration of a crude extract from the anterior lobe of the pituitary gland to a normal rat increases the content of insulin in the pancreas to nearly twice the normal value but when such an extract is injected into a dog there may be a fall in the insulin content of the dog's pancreas (354). In the dog the blood sugar content changes in opposite direction to the insulin content of the pancreas. When permanent diabetes has been established the content of insulin in the pancreas is reduced almost to zero and this condition holds throughout 198 days (355). Attempts have been made to fractionate the hormone from the extract of the pituitary which is responsible for the diabetogenic activity but the results are not yet definite (356). Dogs made permanently diabetic by treatment with an extract of the anterior pituitary differ somewhat from depancreatized dogs. They may require more insulin for control of the glycosuria but they are not abnormally insensitive to insulin. They survive for long periods in good health without insulin, provided that sufficient of the ingested food can be utilized. A high protein diet causes hyperglycemia, ketonuria, and glycosuria. The dogs' reactions to changes in intake of fat, carbohydrate, and protein indicate that protein and not fat is particularly concerned in the causation of ketonuria: substitution of raw meat for casein in the diet increases the ketonuria. The metabolic rate of dogs made diabetic with the pituitary extract is greater than normal but not as high as that of depancreatized dogs (357). In hypophysectomized rats the insulin content of the pancreas is slightly less than in normal animals, and it is further reduced by a diet rich in fat (358).

In dogs made diabetic by the injection of an extract of the pituitary there is no doubt that the disturbance in carbohydrate metabolism is due to degeneration in the islands of Langerhans (359), and in cats a similar study has shown that permanent diabetes cannot be produced in the presence of the intact pancreas, but if from a half to three

fourths of the pancreas is removed and the animal is then treated with an extract from the anterior pituitary gland, the animal becomes permanently diabetic. Characteristic histologic changes in the islands of Langerhans are associated with the temporary and permanent diabetic states (360).

The Effect of Estrogens and Androgens on the Pituitary Gland

The mutual relationship between the anterior pituitary gland and the gonads is shown by the absence of growth of the pituitary gland in ovariectomized rats in contrast to the prolonged growth of the pituitary as indicated by definite and sustained mitotic reactions to colchicine after injection of estrogens (361).

The damage to the sex glands and the influence on cyclic changes in the female genital tract and in the urinary excretion of gonadotropic hormones produced by steroid hormones do not appear to be a direct action on the gonads but are exerted indirectly through the pituitary gland (362, 363, 364, 365).

The present interpretation of the significance and mode of action of the antihormones (366, 367, 368) and a comprehensive summary of our knowledge of the pituitary body (369) have been published.

LITERATURE CITED

1. LERMAN, J., AND SALTER, W. T., *Endocrinology,* **25**, 712 (1939)
2. LUDWIG, W., AND MUTZENBECHER, P. VON, *Z. physiol. Chem.,* **258**, 195 (1939)
3. MUTZENBECHER, P. VON, *Z. physiol. Chem.,* **261**, 253 (1939)
4. BLOCK, JR., P., *J. Biol. Chem.,* **135**, 51 (1940)
5. FOSTER, G. L., PALMER, W. W., AND LELAND, J. P., *J. Biol. Chem.,* **115**, 467 (1936)
6. CANZANELLI, A., GUILD, R., AND RAPPORT, D., *Endocrinology,* **25**, 707 (1939)
7. STELLAR, L. I., AND OLKEN, H. G., *Endocrinology,* **27**, 614 (1940)
8. WHITESIDE, B., *Endocrinology,* **26**, 136 (1940)
9. STERNHEIMER, R., *Endocrinology,* **25**, 899 (1939)
10. SCHUMANN, H., *Z. ges. exptl. Med.,* **105**, 577 (1939)
11. BRENTANO, C., *Z. klin Med.,* **135**, 495 (1939)
12. SMITH, D. C., AND PERMAN, J. M., *Endocrinology,* **27**, 110 (1940)
13. CUTTING, W. C., AND KUZELL, W. C., *J. Pharmacol.,* **69**, 37 (1940)
14. SHERWOOD, T. C., *Endocrinology,* **26**, 693 (1940)
15. SHERWOOD, T. C., *Endocrinology,* **27**, 925 (1940)
16. ALTHAUSEN, T. L., LOCKHART, J. C., AND SOLEY, M. H., *Am. J. Med. Sci.,* **199**, 342 (1940)
17. KLEIN, J. R., *J. Biol. Chem.,* **131**, 139 (1939)
18. SOFFER, L. J., GROSSMAN, E., AND SOBOTKA, H., *J. Biol. Chem.,* **133**, xcii (1940)
19. ABELIN, I., *Nature,* **145**, 935 (1940)
20. SCHACHTER, R. J., AND HUNTINGTON, J., *Proc. Soc. Exptl. Biol. Med.,* **44**, 66 (1940)
21. REICHSTEIN, T., AND FUCHS, H. G., *Helv. Chim. Acta,* **23**, 676 (1940)
22. SHOPPEE, C. W., *Helv. Chim. Acta,* **23**, 740 (1940)
23. SHOPPEE, C. W., AND REICHSTEIN, T., *Helv. Chim. Acta,* **23**, 729 (1940)
24. REICHSTEIN, T., AND SCHINDLER, W., *Helv. Chim. Acta,* **23**, 669 (1940)
25. REICHSTEIN, T., AND FUCHS, H. G., *Helv. Chim. Acta,* **23**, 658 (1940)
26. REICHSTEIN, T., AND FUCHS, H. G., *Helv. Chim. Acta,* **23**, 684 (1940)
27. MAMOLI, L., *Ber.,* **72**, 1863 (1939)
28. RUZICKA, L., AND MELDAHL, H. F., *Helv. Chim. Acta,* **23**, 364 (1940)
29. PLATTNER, P. A., AND SCHRECK, W., *Helv. Chim. Acta,* **22**, 1178 (1939)
30. STAVELY, H. E., *J. Am. Chem. Soc.,* **62**, 489 (1940)
31. GOLDBERG, M. W., AND AESCHBACHER, R., *Helv. Chim. Acta,* **22**, 1185 (1939)
32. PFIFFNER, J. J., AND NORTH, H. B., *J. Biol. Chem.,* **132**, 459 (1940)
33. CARNES, W. H., *Proc. Soc. Exptl. Biol. Med.,* **45**, 502 (1940)
34. SPURR, C. L., AND KOCHAKIAN, C. D., *Endocrinology,* **25**, 782 (1939)
35. PFEIFFER, C. A., AND HOOKER, C. W., *Am. J. Physiol.,* **131**, 441 (1940)
36. COREY, E. L., *Am. J. Physiol.,* **129**, P340 (1940)
37. EMERY, F. E., AND GRECO, P. A., *Endocrinology,* **27**, 473 (1940)
38. KUIZENGA, M. H., AND CARTLAND, G. F., *Endocrinology,* **27**, 647 (1940)
39. WETTSTEIN, A., AND HUNZIKER, F., *Helv. Chim. Acta,* **23**, 764 (1940)

40. Zwemer, R. L., Lowenstein, B. E., and Pines, K. L., *Endocrinology,* **27**, 945 (1940)
41. Dorfman, R. I., *Proc. Soc. Exptl. Biol. Med.,* **43**, 489 (1940)
42. Kuizenga, M. H., Nelson, J. W., and Cartland, G. F., *Am. J. Physiol.,* **130**, 298 (1940)
43. Uyldert, I. E., *Endocrinology,* **25**, 871 (1939)
44. Uyldert, I. E., Thomassen, J., and Waterman, L., *Endocrinology,* **25**, 877 (1939)
45. Waterman, L., Uyldert, I. E., Thomassen, J., and Oestreicher, F., *Endocrinology,* **25**, 885 (1939)
46. Waterman, L., *Arch. intern. pharmacodynamie,* **64**, 46 (1940)
47. Anderson, E., Joseph, M., and Herring, V. V., *Proc. Soc. Exptl. Biol. Med.,* **44**, 477 (1940)
48. Anderson, E., Joseph, M., and Herring, V. V., *Proc. Soc. Exptl. Biol. Med.,* **44**, 482 (1940)
49. Cleghorn, R. A., Armstrong, C. W. J., and Austen, D. C., *Endocrinology,* **25**, 888 (1939)
50. Dennis, C., and Wood, E. H., *Am. J. Physiol.,* **129**, 182 (1940)
51. Muntwyler, E., Mellors, R. C., and Mautz, F. R., *J. Biol. Chem.,* **134**, 345 (1940)
52. Muntwyler, E., Mellors, R. C., Mautz, F. R., and Mangun, G. H., *J. Biol. Chem.,* **134**, 367 (1940)
53. Remington, J. W., *Endocrinology,* **26**, 631 (1940)
54. Cantarow, A., and Rakoff, A. E., *Endocrinology,* **27**, 652 (1940)
55. Menkin, V., *Am. J. Physiol.,* **129**, 691 (1940)
56. Campbell, C. H., *J. Okla. State Med. Assoc.,* **32**, 447 (1939)
57. Truszkowski, R., and Duszynska, J., *Endocrinology,* **27**, 117 (1940)
58. Pulver, R., and Verzár, F., *Helv. Chim. Acta,* **23**, 1087 (1940)
59. Lasnitzki, A., *Nature,* **146**, 99 (1940)
60. Cicardo, V. H., and Moglia, J. A., *Nature,* **145**, 551 (1940)
61. Verzár, F., and Somogyi, J. C., *Nature,* **145**, 781 (1940)
62. Miller, H. C., and Darrow, D. C., *Am. J. Physiol.,* **129**, 264 (1940)
63. Nelson, N., Grayman, I., and Mirsky, I. A., *J. Biol. Chem.,* **132**, 711 (1940)
64. Shipley, R. A., *Endocrinology,* **26**, 900 (1940)
65. Karády, S., Rose, B., and Browne, J. S. L., *Am. J. Physiol.,* **130**, 539 (1940)
66. Crismon, J. M., and Field, II, J., *Am. J. Physiol.,* **130**, 231 (1940)
67. Russell, J. A., and Wilhelmi, A. E., *Am. J. Physiol.,* **129**, P453 (1940)
68. Harris, R. E., and Ingle, D. J., *Am. J. Physiol.,* **130**, 151 (1940)
69. Ingle, D. J., *Endocrinology,* **26**, 478 (1940)
70. Winter, C. A., and Knowlton, G. C., *Am. J. Physiol.,* **131**, 465 (1940)
71. Ingle, D. J., *Am. J. Physiol.,* **129**, 278 (1940)
72. Fowler, J. L. A., Clarke, A. P. W., and Cleghorn, R. A., *Am. J. Physiol.,* **129**, P356 (1940)
73. Waterman, L., Danby, M., Gaarenstroom, J. H., Spanhoff, R. W., and Uyldert, I. E., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.,* **9**, 75 (1939)

74. HARTMAN, F. A., AND SPOOR, H. J., *Endocrinology,* 26, 871 (1940)
75. HARTMAN, F. A., LEWIS, L. A., GABRIEL, J. E., SPOOR, H. J., AND BROWNELL, K. A., *Endocrinology,* 27, 287 (1940)
76. HARTMAN, F. A., AND DUBACH, R., *Endocrinology,* 27, 638 (1940)
77. HARTMAN, F. A., BROWNELL, K. A., WALTHER, R., AND EDELMANN, A., *Endocrinology,* 27, 642 (1940)
78. KENDALL, E. C., *Proc. Staff Meetings Mayo Clinic,* 15, 297 (1940)
79. JUHLMANN, D., RAGAN, C., FERREBEE, J. W., ATCHLEY, D. W., AND LOEB, R. F., *Science,* n.s. 90, 496 (1939)
80. WELLS, B. B., AND KENDALL, E. C., *Proc. Staff Meetings Mayo Clinic,* 15, 133 (1940)
81. RAGAN, C., FERREBEE, J. W., PHYFE, P., ATCHLEY, D. W., AND LOEB, R. F., *Am. J. Physiol.,* 131, 73 (1940)
82. WELLS, B. B., AND KENDALL, E. C., *Proc. Staff Meetings Mayo Clinic,* 15, 324 (1940)
83. INGLE, D. J., *Proc. Soc. Exptl. Biol. Med.,* 44, 174 (1940)
84. WATSON, E. M., *Endocrinology,* 27, 521 (1940)
85. LEVIE, L. H., AND UYLDERT, I. E., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.,* 9, 121 (1939)
86. CUYLER, W. K., ASHLEY, C., AND HAMBLEN, E. C., *Endocrinology,* 27, 177 (1940)
87. CLAUSEN, H. J., *Endocrinology,* 27, 989 (1940)
88. COREY, E. L., AND BRITTON, S. W., *Am. J. Physiol.,* 129, P340 (1940)
89. SPRAGUE, R. G., *Proc. Staff Meetings Mayo Clinic,* 15, 291 (1940)
90. LONG, C. N. H., KATZIN, B., AND FRY, E. G., *Endocrinology,* 26, 309 (1940)
91. GRATTAN, J. F., AND JENSEN, H., *J. Biol. Chem.,* 135, 511 (1940)
92. THORN, G. W., KOEPF, G. F., LEWIS, R. A., AND OLSEN, E. F., *J. Clin. Investigation,* 19, 813 (1940)
93. SELYE, H., AND DOSNE, C., *Am. J. Physiol.,* 128, 729 (1940)
94. INGLE, D. J., *Proc. Soc. Exptl. Biol. Med.,* 44, 176 (1940)
95. WELLS, B. B., AND KENDALL, E. C., *Proc. Staff Meetings Mayo Clinic,* 15, 565 (1940)
96. WELLS, B. B., *Proc. Staff Meetings Mayo Clinic,* 15, 294 (1940)
97. WELLS, B. B., AND KENDALL, E. C., *Proc. Staff Meetings Mayo Clinic,* 15, 493 (1940)
98. WELLS, B. B., AND CHAPMAN, A., *Proc. Staff Meetings Mayo Clinic,* 15, 503 (1940)
99. INGLE, D. J., *Endocrinology,* 26, 472 (1940)
100. INGLE, D. J., *Endocrinology,* 27, 297 (1940)
101. ETTELSON, L. N., *Endocrinology,* 27, 340 (1940)
102. GALLI, T., AND SABATELLI, F., *Pathologica,* 32, 113 (1940)
103. PERLA, D., FREIMAN, D. G., SANDBERG, M., AND GREENBERG, S. S., *Proc. Soc. Exptl. Biol. Med.,* 43, 397 (1940)
104. SELYE, H., DOSNE, C., BASSETT, L., AND WHITTAKER, J., *Can. Med. Assoc. J.,* 43, 1 (1940)
105. SELYE, H., AND DOSNE, C., *Lancet,* II, 70 (1940)
106. FINE, J., FUCHS, F., AND MARK, J., *Proc. Soc. Exptl. Biol. Med.,* 43, 514 (1940)

107. RAGAN, C., FERREBEE, J. W., AND FISH, G. W., *Proc. Soc. Exptl. Biol. Med.,* **42**, 712 (1939)
108. LONG, C. N. H., *J. Am. Dietet. Assoc.,* **16**, 395 (1940)
109. RUSSELL, J. A., *Am. J. Physiol.,* **128**, 552 (1940)
110. FERREBEE, J. W., RAGAN, C., ATCHLEY, D. W., AND LOEB, R. F., *Endocrinology,* **27**, 360 (1940)
111. THORN, G. W., AND FIROR, W. M., *J. Am. Med. Assoc.,* **114**, 2517 (1940)
112. THADDEA, S., AND SARKANDER, H., *Z. klin. Med.,* **136**, 267 (1939)
113. MOEHLIG, R. C., *Endocrinology,* **27**, 633 (1940)
114. McCULLAGH, E. P., AND RYAN, A. J., *J. Am. Med. Assoc.,* **114**, 2530 (1940)
115. BORNE, G. A. K. VON DEM, AND CARDOZO, E. L., *Nederland. Tijdschr. Geneeskunde,* **84**, 231 (1940); *Chem. Abstracts,* **34**, 2457 (1940)
116. BLOOMFIELD, A. L., *Bull. Johns Hopkins Hosp.,* **65**, 456 (1939)
117. LOEB, R. F., *Bull. N. Y. Acad. Med.,* **16**, 347 (1940)
118. SOFFER, L. J., ENGLE, F. L., AND OPPENHEIMER, B. S., *J. Am. Med. Assoc.,* **115**, 1860 (1940)
119. WILDER, R. M., *Proc. Staff Meetings, Mayo Clinic,* **15**, 273 (1940)
120. TOOKE, JR., T. B., POWER, M. H., AND KEPLER, E. J., *Proc. Staff Meetings, Mayo Clinic,* **15**, 365 (1940)
121. GORDON, E. S., *J. Am. Med. Assoc.,* **114**, 2549 (1940)
122. PERLA, D., AND MARMORSTON, J., *Endocrinology,* **27**, 367 (1940)
123. COCKERELL, T. D. A., *Science,* n. s. **92**, 173 (1940)
124. VERZÁR, F., *Die Funktion der Nebennierenrinde,* 266 pp. (Benno Schwabe & Co. Verlag, Basel, 1939)
125. BRUCE, H. M., AND WIEN, R., *J. Physiol.,* **98**, 375 (1940)
126. NELSON, D., *Am. J. Physiol.,* **129**, P429 (1940)
127. OCHOA, S., AND ROSSITER, R. J., *J. Physiol.,* **97**, 1P (1939–40)
128. RUNNSTRÖM, J., SPERBER, E., AND BÁRÁNY, E., *Nature,* **145**, 106 (1940)
129. HOUSSAY, B. A., FOGLIA, V. G., AND FUSTINONI, O., *Rev. soc. argentina biol.,* **15**, 139 (1940)
130. MARRAZZI, R., *Am. J. Physiol.,* **131**, 36 (1940)
131. ANDERSON, E., JOSEPH, M., AND HERRING, V. V., *Proc. Soc. Exptl. Biol. Med.,* **42**, 782 (1939)
132. ANDERSON, E., HERRING, V. V., AND JOSEPH, M., *Proc. Soc. Exptl. Biol. Med.,* **45**, 488 (1940)
133. LEWIS, R. A., KUHLMAN, D., DELBUE, C., KOEPF, G. F., AND THORN, G. W., *Endocrinology,* **27**, 971 (1940)
134. SWANN, H. G., *Physiol. Rev.,* **20**, 493 (1940)
135. EHRENSTEIN, M., AND STEVENS, T. O., *J. Biol. Chem.,* **133**, xxvii (1940)
136. EHRENSTEIN, M., AND STEVENS, T. O., *J. Org. Chem.,* **5**, 318 (1940)
137. MARKER, R. E., AND ROHRMANN, E., *J. Am. Chem. Soc.,* **62**, 898 (1940)
138. MARKER, R. E., AND ROHRMANN, E., *J. Am. Chem. Soc.,* **62**, 518 (1940)
139. KIMURA, T., AND SUGIYAMA, G., *J. Biochem. (Japan),* **29**, 409 (1939)
140. MARKER, R. E., AND KRUEGER, J., *J. Am. Chem. Soc.,* **62**, 79 (1940)
141. HASKINS, A. L., *Endocrinology,* **27**, 983 (1940)
142. SZARKA, A. J., *J. Endocrinol.,* **2**, 1 (1940)
143. STEINKAMM, E., *Arch. Gynäkol.,* **169**, 53 (1939)
144. GILLMAN, J., *Endocrinology,* **26**, 1072 (1940)

145. GILLMAN, J., *Endocrinology,* **26,** 80 (1940)
146. ALLENDE, I. L. C., *Proc. Soc. Exptl. Biol. Med.,* **44,** 534 (1940)
147. HAMBLEN, E. C., CUYLER, W. K., AND HIRST, D. V., *Endocrinology,* **27,** 33 (1940)
148. MÜLLER, H. A., *Klin. Wochschr.,* **19,** 318 (1940)
149. VENNING, E. H., AND BROWNE, J. S. L., *Endocrinology,* **27,** 707 (1940)
150. HAMBLEN, E. C., CUYLER, W. K., AND HIRST, D. V., *Endocrinology,* **27,** 172 (1940)
151. HAMBLEN, E. C., CUYLER, W. K., AND HIRST, D. V., *Endocrinology,* **27,** 169 (1940)
152. WESTPHAL, U., AND BUXTON, C. L., *Proc. Soc. Exptl. Biol. Med.,* **42,** 749 (1939)
153. MARKER, R. E., AND HARTMAN, C. G., *J. Biol. Chem.,* **133,** 529 (1940)
154. BUCHER, N. L. R., AND GESCHICKTER, C. F., *Endocrinology,* **27,** 727 (1940)
155. LEATHEM, J. H., AND CRAFTS, R. C., *Endocrinology,* **27,** 283 (1940)
156. GREENE, R. R., BURRILL, M. W., AND THOMSON, D. M., *Endocrinology,* **27,** 469 (1940)
157. MAZER, C., AND ISRAEL, S. L., *Am. J. Obstet. Gynecol.,* **38,** 625 (1939)
158. SELYE, H., AND FRIEDMAN, S. M., *Endocrinology,* **27,** 857 (1940)
159. SELYE, H., *Anat. Record,* **75,** 59 (1939)
160. KUNZ, A. C., *Rev. sud-americana endocrinol. inmunol. quimioterap.,* **23,** 123 (1940); *Chem. Abstracts,* **34,** 3800 (1940)
161. SELYE, H., *Proc. Soc. Exptl. Biol. Med.,* **43,** 343 (1940)
162. KLEINER, I. S., *Endocrinology,* **26,** 534 (1940)
163. EMMENS, C. W., AND PARKES, A. S., *J. Endocrinol.,* **1,** 332 (1939)
164. SALMON, U. J., AND SALMON, A. A., *Proc. Soc. Exptl. Biol. Med.,* **43,** 709 (1940)
165. HAMBLEN, E. C., CUYLER, W. K., AND HIRST, D. V., *Endocrinology,* **27,** 35 (1940)
166. HAMBLEN, E. C., POWELL, N. B., CUYLER, W. K., AND PATTEE, C. J., *Endocrinology,* **26,** 201 (1940)
167. BACHMANN, W. E., COLE, W., AND WILDS, A. L., *J. Am. Chem. Soc.,* **62,** 824 (1940)
168. BACHMANN, W. E., AND WILDS, A. L., *J. Am. Chem. Soc.,* **62,** 2084 (1940)
169. PEARLMAN, W. H., AND WINTERSTEINER, O., *J. Biol. Chem.,* **132,** 605 (1940)
170. LONGWELL, B., AND WINTERSTEINER, O., *J. Biol. Chem.,* **133,** 219 (1940)
171. HUFFMAN, M. N., MacCORQUODALE, D. W., THAYER, S. A., DOISY, E. A., SMITH, G. V., AND SMITH, O. W., *J. Biol. Chem.,* **134,** 591 (1940)
172. DAVID, K. G., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.,* **10,** 30 (1940)
173. HUFFMAN, M. N., THAYER, S. A., AND DOISY, E. A., *J. Biol. Chem.,* **133,** 567 (1940)
174. HEARD, R. D. H., AND HOFFMAN, M. M., *J. Biol. Chem.,* **135,** 801 (1940)
175. MÜHLBOCK, O., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.,* **10,** 1 (1940)

176. Mayer, D. T., Andrews, F. N., and McKenzie, F. F., *Endocrinology,* 27, 867 (1940)
177. Marlow, H. W., and Richert, D., *Endocrinology,* 26, 531 (1940)
178. Beall, D., *J. Endocrinol.,* 2, 81 (1940)
179. Habedorn, A., Johannessohn, F., Rabald, E., and Voss, H. E., *Z. physiol. Chem.,* 264, 23 (1940)
180. Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., and Butler, A. M., *J. Biol. Chem.,* 134, 319 (1940)
181. Szego, C. M., and Samuels, L. T., *Proc. Soc. Exptl. Biol. Med.,* 43, 263 (1940)
182. Mühlbock, O., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.* 10, 42 (1940)
183. Fish, W. R., and Dorfman, R. I., *Science,* 91, 388 (1940)
184. Heller, C. G., *Endocrinology,* 26, 619 (1940)
185. Glass, S. J., Edmondson, H. A., and Soll, S. N., *Endocrinology,* 27, 749 (1940)
186. Pincus, G., and Martin, D. W., *Endocrinology,* 27, 838 (1940)
187. Westerfield, W. W., *Biochem. J.,* 34, 51 (1940)
188. Holden, R. B., *Endocrinology,* 25, 593 (1939)
189. Hechter, O., Lev, M., and Soskin, S., *Endocrinology,* 26, 73 (1940)
190. Astwood, E. B., *Am. J. Physiol.,* 129, P302 (1940)
191. Greene, R. R., and Harris, S. C., *Proc. Soc. Exptl. Biol. Med.,* 45, 34 (1940)
192. Kerly, M., *Biochem. J.,* 34, 814 (1940)
193. Pincus, G., and Graubard, M., *Endocrinology,* 26, 684 (1940)
194. Talbot, N. B., Lowry, O. H., and Astwood, E. B., *J. Biol. Chem.,* 132, 1 (1940)
195. Arenas, N., and Sammartino, R., *Bull. histol. appl. physiol. path. tech. microscop.,* 16, 229 (1939)
196. Arenas, N., and Sammartino, R., *Rev. soc. argentina biol.,* 15, 278 (1940)
197. Sammartino, R., and Arenas, N., *Rev. soc. argentina biol.,* 16, 290 (1940)
198. Sammartino, R., and Arenas, N., *Rev. soc. argentina biol.,* 15, 51 (1939)
199. Paola, G. di, *Rev. soc. argentina biol.,* 15, 61 (1939)
200. Castillo, E. B. del, and Paola, G. di, *Rev. soc. argentina biol.,* 15, 434 (1939)
201. Pattee, C. J., Venning, E. H., and Browne, J. S. L., *Endocrinology,* 27, 721 (1940)
202. Gaarenstroom, J. H., *J. Exptl. Zoöl.,* 82, 31 (1939)
203. Gaarenstroom, J. H., *J. Endocrinol.,* 2, 47 (1940)
204. Foote, C. L., *Proc. Soc. Exptl. Biol. Med.,* 43, 519 (1940)
205. Ackart, R. J., and Leavy, S., *Proc. Soc. Exptl. Biol. Med.,* 42, 720 (1939)
206. Sutro, C. J., *Proc. Soc. Exptl. Biol. Med.,* 44, 151 (1940)
207. Wentworth, J. H., Smith, P. K., and Gardner, W. U., *Endocrinology,* 26, 61 (1940)
208. Lipschütz, A., and Vargas, L., *Lancet,* I, 1313 (1939)
209. Noble, R. L., McEuen, C. S., and Collip, J. B., *Can. Med. Assoc. J.,* 42, 413 (1940)

210. MOHS, F. E., *Proc. Soc. Exptl. Biol. Med.,* **43,** 270 (1940)

211. PINCUS, G., AND GRAUBARD, M., *Endocrinology,* **26,** 427 (1940)

212. MARLOW, H. W., AND RICHERT, D., *Endocrinology,* **27,** 274 (1940)

213. AVERY, T. B., SCOTT, H. M., AND CONRAD, R. M., *Endocrinology,* **27,** 83 (1940)

214. LYONS, W. R., AND SAKO, Y., *Proc. Soc. Exptl. Biol. Med.,* **44,** 398 (1940)

215. SPEERT, H., *Science,* **92,** 461 (1940)

216. SELYE, H., *Am. J. Physiol.,* **130,** 358 (1940)

217. BURNS, E. L., AND SCHENKEN, J. R., *Proc. Soc. Exptl. Biol. Med.,* **40,** 497 (1939)

218. BROYLES, E. N., *Bull. Johns Hopkins Hosp.,* **66,** 319 (1940)

219. EHRENSTEIN, M., *Am. J. Pharm.,* **112,** 7 (1940)

220. SEVRINGHAUS, E. L., "Diseases of the sex glands," in *Modern Medical Therapy in General Practice,* (BARR, D. P.), Vol. 3, p. 3161 (The Williams & Wilkins Company, 1940)

221. SEVRINGHAUS, E. L., *Bull. N.Y. Acad. Med.,* **16,** 53 (1940)

222. CAMPBELL, N. R., DODDS, E. C., AND LAWSON, W., *Proc. Roy Soc. (London), B,* **128,** 253 (1940)

223. DOCKEN, A. M., AND SPIELMAN, M. A., *J. Am. Chem. Soc.,* **62,** 2163 (1940)

224. WESSELY, F. V., AND KLEEDORFER, A., *Naturwissenschaften,* **27,** 567 (1939)

225. CAMPBELL, N. R., DODDS, E. C., LAWSON, W., AND NOBLE, R. L., *Lancet,* **II,** 312 (1939)

226. DINGEMANSE, E., *Nature,* **145,** 825 (1940)

227. NOBLE, R. L., *J. Endocrinol.,* **1,** 216 (1939)

228. GRUMBRECHT, P., AND LOESER, A., *Arch. exptl. Path. Pharmakol.,* **193,** 34 (1939)

229. WILLIAMS, P. C., *Nature,* **145,** 388 (1940)

230. MELLISH, C. H., BAER, A. J., AND MACIAS, A. C., *Endocrinology,* **26,** 273 (1940)

231. NOBLE, R. L., *J. Endocrinol.,* **1,** 128 (1939)

232. GAARENSTROOM, J. H., AND LEVIE, L. H., *J. Endocrinol.,* **1,** 420 (1939)

233. FOLLEY, S. J., WATSON, H. M. S., AND BOTTOMLEY, A. C., *J. Physiol.,* **98,** 15P (1940)

234. ENTENMAN, C., LORENZ, F. W., AND CHAIKOFF, I. L., *J. Biol. Chem.,* **134,** 495 (1940)

235. LISSER, H., *Endocrinology,* **27,** 385 (1940)

236. WESSELY, F. V., *Angew. Chem.,* **53,** 197 (1940); *Chem. Abstracts,* **34,** 6683 (1940)

237. WEINSTEIN, B. B., WEED, J. C., LOCK, F. R., AND COLLINS, C. G., *Endocrinology,* **27,** 739 (1940)

238. PAYNE, S., AND SHELTON, E. K., *Endocrinology,* **27,** 45 (1940)

239. KURZROK, R., WILSON, L., AND PERLOFF, W. H., *Endocrinology,* **26,** 581 (1940)

240. ATKINSON, D. W., *Endocrinology,* **27,** 161 (1940)

241. BISHOP, P. M. F., BOWES, R. K., BOYCOTT, M., KELLAR, R., MACGREGOR, T. N., AND MURLESS, B. C., *Lancet,* **I,** 629 (1940)

242. MACBRYDE, C. M., FREEDMAN, H., LOEFFEL, E., AND ALLEN, D., *Proc. Soc. Exptl. Biol. Med.,* **43,** 212 (1940)

243. INHOFFEN, H. H., ZÜHLSDORFF, G., AND HUANG-MINLON, *Ber.*, **73**, 451 (1940)
244. MADAEVA, O. S., USHAKOV, M. I., AND KOSHELEVA, N. F., *J. Gen. Chem. (U.S.S.R.)*, **10**, 213 (1940); *Chem. Abstracts*, **34**, 7292 (1940)
245. CONSOLAZIO, W. V., AND TALBOTT, J. H., *Endocrinology*, **27**, 355 (1940)
246. BAUMANN, E. J., AND METZGER, N., *Endocrinology*, **27**, 664 (1940)
247. TALBOT, N. B., BUTLER, A. M., AND MACLACHLAN, E. A., *J. Biol. Chem.*, **132**, 595 (1940)
248. TALBOT, N. B., BUTLER, A. M., MACLACHLAN, E. A., AND JONES, R. N., *J. Biol. Chem.*, **136**, 365 (1940)
249. FRIEDGOOD, H. B., AND WHIDDEN, H. L., *Endocrinology*, **27**, 258 (1940)
250. FRIEDGOOD, H. B., AND WHIDDEN, H. L., *Endocrinology*, **27**, 249 (1940)
251. FRIEDGOOD, H. B., AND WHIDDEN, H. L., *Endocrinology*, **27**, 242 (1940)
252. MCCULLAGH, D. R., SCHNEIDER, I., AND EMERY, F., *Endocrinology*, **27**, 71 (1940)
253. DRIPS, D. G., AND OSTERBERG, A. E., *Endocrinology*, **27**, 345 (1940)
254. HOLTORFF, A. F., AND KOCH, F. C., *J. Biol. Chem.*, **135**, 377 (1940)
255. NELSON, D., AND GREENE, R. R., *Endocrinology*, **26**, 824 (1940)
256. TISLOWITZ, R., *Endocrinology*, **25**, 749 (1939)
257. FLEISCHMANN, W., *Endocrinology*, **25**, 798 (1939)
258. EMMENS, C. W., *Med. Research Council (Brit.) Special Rept. Series*, No. 234, 71 pp. (1939)
259. DORFMAN, R. I., AND HAMILTON, J. B., *J. Biol. Chem.*, **133**, 753 (1940)
260. DORFMAN, R. I., *J. Biol. Chem.*, **132**, 457 (1940)
261. DORFMAN, R. I., AND FISH, W. R., *J. Biol. Chem.*, **135**, 349 (1940)
262. BURRILL, M. W., AND GREENE, R. R., *Proc. Soc. Exptl. Biol. Med.*, **44**, 273 (1940)
263. BISKIND, G. R., *Proc. Soc. Exptl. Biol. Med.*, **43**, 259 (1940)
264. HOOKER, C. W., AND COLLINS, V. J., *Endocrinology*, **26**, 269 (1940)
265. DANBY, M., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **10**, 56 (1940)
266. GREENE, R. R., AND BURRILL, M. W., *Proc. Soc. Exptl. Biol. Med.*, **43**, 382 (1940)
267. SPEERT, H., *Bull. Johns Hopkins Hosp.*, **67**, 189 (1940)
268. MAZER, M., AND MAZER, C., *Endocrinology*, **26**, 662 (1940)
269. HARTMAN, C. G., *Endocrinology*, **26**, 449 (1940)
270. GREENE, R. R., AND BURRILL, M. W., *Proc. Soc. Exptl. Biol. Med.*, **42**, 585 (1939)
271. STEINKAMM, E., AND MECKIES, H., *Arch. Gynäkol.*, **168**, 436 (1939)
272. HERRICK, E. H., AND LOCKHART, C. H., *Endocrinology*, **26**, 508 (1940)
273. SELYE, H., *Endocrinology*, **27**, 657 (1940)
274. SELYE, H., *Anat. Record*, **76**, 145 (1940)
275. FOOTE, C. L., AND WITSCHI, E., *Anat. Record*, **75**, 75 (1939)
276. BALDWIN, F. M., AND GOLDIN, H. S., *Proc. Soc. Exptl. Biol. Med.*, **42**, 813 (1939)
277. PASCHKIS, K. E., SHAY, H., GERSHON-COHEN, J., AND FELS, S. S., *Am. J. Physiol.*, **129**, 191 (1940)
278. GAARENSTROOM, J. H., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **9**, 134 (1939)

279. HARSH, R., OVERHOLSER, M. D., AND WELLS, L. J., *J. Endocrinol.*, 1, 261 (1939)

280. GREENE, R. R., AND BURRILL, M. W., *Am. J. Physiol.*, 129, P368 (1940)

281. ROTHERMICH, N. O., *Endocrinology*, 25, 520 (1939)

282. HARRIS, S. C., AND GREENE, R. R., *Am. J. Physiol.*, 129, P377 (1940)

283. NOBLE, G. K., AND WURM, M., *Endocrinology*, 26, 837 (1940)

284. WITSCHI, E., AND FUGO, N. W., *Proc. Soc. Exptl. Biol. Med.*, 45, 10 (1940)

285. RUBIN, I. C., AND DAVIDS, A. M., *Endocrinology*, 26, 523 (1940)

286. BURDICK, H. O., EMERSON, B. B., AND WHITNEY, R., *Endocrinology*, 26, 1081 (1940)

287. WILSON, L., AND KURZROK, R., *Endocrinology*, 26, 587 (1940)

288. GARDNER, W. U., AND VAN HEUVERSWYN, J., *Endocrinology*, 26, 833 (1940)

289. DICK, M., AND HOOKER, C. W., *J. Lab. Clin. Med.*, 25, 33 (1939)

290. KOCHAKIAN, C. D., *Endocrinology*, 26, 54 (1940)

291. EMMENS, C. W., AND BRADSHAW, T. E. T., *J. Endocrinol.*, 1, 378 (1939)

292. MACKAY, E. M., *Proc. Soc. Exptl. Biol. Med.*, 45, 216 (1940)

293. SELYE, H., *J. Urol.*, 42, 637 (1939)

294. SELYE, H., *J. Pharmacol.*, 68, 454 (1940)

295. CHIODI, H., *Endocrinology*, 26, 107 (1940)

296. CHIODI, H., *Rev. soc. argentina biol.*, 14, 309 (1938)

297. RAPFOGEL, I., *Endocrinology*, 27, 179 (1940)

298. McCULLAGH, E. P., AND McGURL, F. J., *Endocrinology*, 26, 377 (1940)

299. SCHUMANN, H., *Klin. Wochschr.*, 19, 364 (1940)

300. KENYON, A. T., KNOWLTON, K., SANDIFORD, I., KOCH, F. C., AND LOTWIN, G., *Endocrinology*, 26, 26 (1940)

301. COFFMAN, J. R., AND KOCH, F. C., *J. Biol. Chem.*, 135, 519 (1940)

302. JAILER, J. W., *Am. J. Physiol.*, 130, 503 (1940)

303. BEILLY, J. S., AND SOLOMON, S., *Endocrinology*, 26, 236 (1940)

304. HAMBLEN, E. C., PATTEE, C. J., AND CUYLER, W. K., *Endocrinology*, 27, 734 (1940)

305. LAWRENCE, C. H., AND WERTHESSEN, N. T., *Endocrinology*, 27, 755 (1940)

306. FRANK, R. T., *J. Am. Med. Assoc.*, 114, 1504 (1940)

307. NATHANSON, I. T., AND TOWNE, L. E., *Endocrinology*, 25, 754 (1939)

308. WERTHESSEN, N. T., AND PINCUS, G., *Am. J. Physiol.*, 129, P494 (1940)

309. McCULLAGH, E. P., AND LILGA, H. V., *Endocrinology*, 26, 753 (1940)

310. CALLOW, N. H., CALLOW, R. K., AND EMMENS, C. W., *J. Endocrinol.*, 2, 88 (1940)

311. HIRSCHMANN, H., *J. Biol. Chem.*, 136, 483 (1940)

312. CALLOW, N. H., AND CALLOW, R. K., *Biochem. J.*, 34, 276 (1940)

313. LI, C. H., SIMPSON, M. E., AND EVANS, H. M., *Endocrinology*, 27, 803 (1940)

314. LI, C. H., SIMPSON, M. E., AND EVANS, H. M., *Science*, 92, 355 (1940)

315. SHEDLOVSKY, T., ROTHEN, A., GREEP, R. O., VAN DYKE, H. B., AND CHOW, B. F., *Science*, 92, 178 (1940)

316. FRAENKEL-CONRAT, H., SIMPSON, M. E., AND EVANS, H. M., *Anales Facultad med. Montevideo*, 25, 3 (1940)

317. JENSEN, H., TOLKSDORF, S., AND BAMMAN, F., *J. Biol. Chem.*, 135, 791 (1940)
318. GREEP, R. O., VAN DYKE, H. B., AND CHOW, B. F., *J. Biol. Chem.*, 133, 289 (1940)
319. GOSS, H., AND COLE, H. H., *Endocrinology*, 26, 244 (1940)
320. COLE, H. H., PENCHARZ, R. I., AND GOSS, H., *Endocrinology*, 27, 548 (1940)
321. GURIN, S., BACHMAN, C., AND WILSON, D. W., *J. Biol. Chem.*, 133, 477 (1940)
322. GURIN, S., BACHMAN, C., AND WILSON, D. W., *J. Biol. Chem.*, 133, 467 (1940)
323. BONSNES, R. W., AND WHITE, A., *Endocrinology,* 26, 990 (1940)
324. FEVOLD, H. L., LEE, M., HISAW, F. L., AND COHN, E. J., *Endocrinology,* 26, 999 (1940)
325. FRAENKEL-CONRAT, J., FRAENKEL-CONRAT, H. L., SIMPSON, M. E., AND EVANS, H. M., *J. Biol. Chem.*, 135, 199 (1940)
326. BATES, R. W., RIDDLE, O., AND MILLER, R. A., *Endocrinology,* 27, 781 (1940)
327. LI, C. H., LYONS, W. R., AND EVANS, H. M., *J. Gen. Physiol.*, 23, 433 (1940)
328. WHITE, A., AND LAVIN, G. I., *J. Biol. Chem.*, 132, 717 (1940)
329. FRAENKEL-CONRAT, H. L., MEAMBER, D. L., SIMPSON, M. E., AND EVANS, H. M., *Endocrinology,* 27, 605 (1940)
330. FREUD, J., AND DINGEMANSE, E., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.,* 10, 102 (1940)
331. LI, C. H., SIMPSON, M. E., AND EVANS, H. M., *J. Biol. Chem.*, 131, 259 (1939)
332. FRAENKEL-CONRAT, H. L., SIMPSON, M. E., AND EVANS, H. M., *Science,* 91, 363 (1940)
333. BOTTOMLEY, A. C., AND FOLLEY, S. J., *Nature,* 145, 304 (1940)
334. JENSEN, H., AND TOLKSDORF, S., *J. Biol. Chem.*, 132, 519 (1940)
335. JENSEN, H., TOLKSDORF, S., AND GRATTAN, J. F., *Am. J. Physiol.*, 128, 532 (1940)
336. CHEN, G., AND VAN DYKE, H. B., *Proc. Soc. Exptl. Biol. Med.,* 40, 172 (1939)
337. GREEP, R. O., *Proc. Soc. Exptl. Biol. Med.,* 42, 454 (1939)
338. CHOW, B. F., GREEP, R. O., AND VAN DYKE, H. B., *J. Endocrinol.,* 1, 440 (1939)
339. DINGEMANSE, E., AND FREUD, J., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.,* 9, 157 (1939)
340. McSHAN, W. H., AND MEYER, R. K., *J. Biol. Chem.*, 135, 473 (1940)
341. McSHAN, W. H., AND MEYER, R. K., *J. Biol. Chem.*, 132, 783 (1940)
342. JENSEN, H., AND GRATTAN, J. F., *Am. J. Physiol.*, 128, 270 (1940)
343. EVANS, J. S., HINES, L. R., CEITHAML, J. J., AND KOCH, F. C., *Endocrinology,* 26, 1012 (1940)
344. BISCHOFF, F., *Endocrinology,* 27, 554 (1940)
345. LEATHEM, J. H., AND WESTPHAL, U., *Endocrinology,* 27, 567 (1940)
346. EVANS, H. M., SIMPSON, M. E., TOLKSDORF, S., AND JENSEN, H., *Endocrinology,* 25, 529 (1939)

347. FRAENKEL-CONRAT, H. L., LI, C. H., SIMPSON, M. E., AND EVANS, H. M., *Endocrinology, 27,* 793 (1940)
348. WITSCHI, E., *Endocrinology, 27,* 437 (1940)
349. WITSCHI, E., AND RILEY, G. M., *Endocrinology,* 26, 565 (1940)
350. EVANS, J. S., HINES, L., VARNEY, R., AND KOCH, F. C., *Endocrinology,* 26, 1005 (1940)
351. MELAMPY, R. M., AND STANLEY, A. J., *Science,* 91, 457 (1940)
352. SEIDLIN, S. M., *Endocrinology,* 26, 696 (1940)
353. COLLIP, J. B., *Am. J. Obstet, Gynecol.,* 39, 187 (1940)
354. MARKS, H. P., AND YOUNG, F. G., *Lancet,* I, 493 (1940)
355. BEST, C. H., CAMPBELL, J., AND HAIST, R. E., *J. Physiol.,* 97, 200 (1940)
356. YOUNG, F. G., *J. Endocrinol.,* 1, 339 (1939)
357. MARKS, H. P., AND YOUNG, F. G., *J. Endocrinol.,* 1, 470 (1939)
358. HAIST, R. E., *J. Physiol.,* 98, 419 (1940)
359. RICHARDSON, K. C., *Proc. Roy. Soc. (London), B,* 128, 153 (1940)
360. LUKENS, F. D. W., AND DOHAN, F. C., *Science,* 92, 222 (1940)
361. KUZELL, W. C., AND CUTTING, W. C., *Endocrinology,* 26, 537 (1940)
362. CHU, J. P., *J. Endocrinol.,* 2, 21 (1940)
363. TURNER, C. D., *J. Exptl. Zoöl.,* 83, 1 (1940)
364. ROTHERMICH, N. O., AND FOLTZ, L. M., *Endocrinology, 27,* 37 (1940)
365. HUFFMAN, J. W., AND BOS, L. H., *Endocrinology,* 26, 259 (1940)
366. NUTTING, M.-D. F., *Endocrinology,* 26, 369 (1940)
367. COLLIP, J. B., SELYE, H., AND THOMSON, D. L., *Biol. Rev. Cambridge Phil. Soc.,* 15, 1 (1940)
368. JAILER, J. W., AND LEATHAM, J. H., *Proc. Soc. Exptl. Biol. Med.,* 45, 506 (1940)
369. RYNEARSON, E. H., AND SCHWEIGER, L. R., *Arch. Internal Med.,* 66, 226 (1940)

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THE WATER-SOLUBLE VITAMINS

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More than seven hundred papers on the water-soluble vitamins were published in 1940. Obviously only a few of these can be reviewed in the space allotted and therefore many valuable and stimulating studies have been regretfully omitted.

Nomenclature.—The roll call of water-soluble vitamins has not yet been completed, since studies in which crystalline vitamins were used have indicated the existence of one or more still unidentified factors. Nevertheless, the time has come when the nearly exhausted alphabet may be left to those unknowns and the known members designated by their proper chemical names. In addition, units of weight—micrograms or milligrams—may now be used for requirements and assays, instead of the confusing "units," International or otherwise, formerly necessary.

THIAMIN

A very large number of papers has again appeared on thiamin and thiamin deficiency. The choice of articles for review has been governed chiefly by their immediate value in practical dietetics.

The outstanding impression gained from review of both experimental and clinical studies of thiamin deficiency is that many of the classical signs and symptoms are due to ancillary deficiencies in factors other than thiamin, perhaps chiefly the members of the B_2 complex, but also protein and vitamin A. Fortification of the diet with thiamin cannot therefore be accepted as anything but a first stumbling step in the right direction.

Assay.—New procedures in biological assay (1, 2) and in microbioassay (3, 4) have been described. The chemical determination of thiamin remains only moderately satisfactory. The two methods generally used are the thiochrome method of Jansen and that involving the use of the McCollum-Prebluda diazotized reagent.

De Caro & Butturini (5) described a fluorometric determination of thiamin and cocarboxylase in tissues with the use of takadiastase for hydrolysis of cocarboxylase. The thiochrome method was modi-

fied by Henry *et al.* for use in milk (6, 7) and by others for use with tissue extracts (8), wheat (9), and urine (10, 11, 12). Schultz *et al.* (13) adapted their fermentation method to use with urines.

The use of the McCollum-Prebluda diazotized reagent for the colorimetric estimation of thiamin has been examined and improved for assay of a variety of products (14, 15, 16). The chief difficulty, in urine studies especially, continues to be the complete removal of interfering substances without accompanying loss of thiamin.

The determination of cocarboxylase.—The most widely used method of estimation of cocarboxylase is based on the production of carbon dioxide when cocarboxylase is added to aetiozymase (alkaline washed yeast), treated with pyruvic acid. Doubt was cast on the accuracy of this method when Ochoa & Peters (17), using baker's yeast, found that free thiamin stimulated carbon dioxide production with a given amount of cocarboxylase, although no synthesis of the vitamin to cocarboxylase could be detected. However, Lipschitz *et al.* (18) and Weil-Malherbe (19) have obtained aetiozymase preparations from brewer's yeast which show no "Ochoa" effect. These contradictory results may in part be explained by the observation (20) that the addition of thiamin (or the pyrimidine portion) inhibits the action of a phosphatase, which if present breaks down the added cocarboxylase.

Lipton & Elvehjem (21) reported the activation of cocarboxylase by thiamin with aetiozymase from brewer's bottom yeast in the presence of hexosediphosphate and boiled tissue extracts. The activation depended upon the type of yeast employed, brewer's yeast showing but little effect. This is given as another possible explanation for the findings of Ochoa & Peters (17). These workers (21) advance the hypothesis that activation by thiamin cannot be due to its synthesis to cocarboxylase, but that the addition of a large excess of thiamin saturates a heat-labile factor in baker's yeast and permits the adsorption of cocarboxylase on the active apoenzyme.

Goodhart & Sinclair's (22) method for blood cocarboxylase has been modified by the use of washed blood cells instead of whole blood and by the addition of 50 instead of 10 µg. of thiamin. The average level in the blood of normal subjects was found to be 7.0 µg. $\pm$ 1.5 S.D. (23). Both cocarboxylase formation and its transport were found to be confined to the blood cells. Westenbrink (24, 25) also modified Ochoa & Peter's method (17), essentially the same as did Goodhart & Sinclair, so as to allow determination of extremely small amounts of thiamin and cocarboxylase.

Widenbauer (26) and also Ritsert (27) described thiochrome methods for the determination of the free thiamin and cocarboxylase of blood and tissues. They also concluded that the total thiamin of normal human blood is not less than 7 μg. per cent.

On the mechanism of cocarboxylase action.—The explanation of the catalytic action of cocarboxylase on pyruvate is in doubt.

Further work on pyruvate oxidation in the brain by Banga *et al.* (28) showed that the reduced power of oxidation in dialyzed brain dispersions could be restored by additions of inorganic phosphate, a C_4 dicarboxylic acid (succinate, fumarate, malate) and "adenosine nucleotide" (adenylic acid or adenosine triphosphate). Citrate, or an intermediate in citric acid metabolism, was much less active than the C_4 dicarboxylic acids, making it seem unlikely that oxidation can take place through a citric acid cycle as suggested by Krebs & Eggleston (29) for muscle.

Sober *et al.* (30) found decreased citric acid excretion and decreased ability to transform succinic acid into citric acid in rats during acute thiamin deficiency. Thiamin therapy increased the citric acid excretion and normal values were restored in ten to fourteen days. It was concluded that thiamin is essential for the synthesis of endogenous citric acid from its precursors but the data did not clarify further the theory of Krebs that citric acid represents the key intermediate in the production of the C_4 dicarboxylic acids from pyruvate. Ochoa (31) more recently showed that under suitable conditions, oxidation of pyruvate in the presence of adenylic acid can cause phosphorylation of both hexosemonophosphate and glucose, "adenine nucleotide" being the intermediate carrier of the phosphate.

Stern & Melnick (32) pointed out that thiamin is not a typical primary amine and that therefore, decarboxylation of pyruvic acid cannot take place by combination with cocarboxylase to form an active substituted imino acid. Lipmann (33) suggested that hydrogen can be added to the quaternary group as in cozymase. Stern & Melnick (32) first reported that the free vitamin can be hydrogenated to dihydrococarboxylase which was said to be active, but on reinvestigation (34) found to be catalytically inactive. However, they believe that enzymatic breakdown of pyruvic acid may involve an oxido-reductive reaction by cocarboxylase.

Barron & Lyman (35) showed *in vitro* that synthesis of carbohydrate and citric acid, with pyruvate as one of the substrates, was diminished in tissues from avitaminotic rats, but increased to normal

on addition of cocarboxylase. They pointed out that the action of thiamin may not be confined only to the oxidation and decarboxylation of pyruvate but may activate pyruvate for all the varied reactions in which it takes part.

Peters (36) has written a good review on cocarboxylase.

Effect of canning and cooking on thiamin.—Lunde *et al.* (37) showed that careful canning procedures may entail losses of less than 10 per cent in vegetables, and that greater losses are due to extraction by the liquor rather than to heat. Aughey & Daniel (38) found that the destruction of thiamin by the boiling of vegetables may amount to as much as 22 per cent and an additional 15 per cent may be lost by solution in the cooking water. Baking of bread caused a 15 per cent loss. Roasting pork caused a loss of 43 per cent, nearly three times the amount of destruction produced by braising. Thermal treatment at 115.6° for 120 min. caused a 70 to 80 per cent loss of thiamin in beef and hog products (39).

The thiamin content of a large number of foods has been estimated by Leong (40) and by Pike (41). In general, meats and unrefined grains appear to be excellent sources. Of the plant foods, nuts and seeds contain the most thiamin; in most leaves the concentration is constant, about 75 μg. per 100 gm., regardless of botanical family.

Radioactive sulfur in thiamin.—Borsook *et al.* (42) made an unusually interesting study of the course of thiamin metabolism in man by use of radioactive sulfur. They injected 16 mg. of thiamin, containing radioactive sulfur, into human subjects daily for four days. In six days after the last injection, 61 per cent of the injected radioactive sulfur was recovered from the urine, 11 per cent from the feces, and 28 per cent was unaccounted for.

Fat formation and thiamin.—Longenecker *et al.* (43) found that rats fed a ration deficient in the vitamin-B complex and free from fat gradually lost fat from their body stores. The addition of 12.5 μg. of thiamin daily resulted in rapid deposition of a characteristic body fat and increase in the total acetone-soluble lipids. Oldham & Schlutz (44) found that with isocaloric intakes, rats receiving 39 μg. of thiamin daily had a greater fat content than litter mates receiving 9 μg. daily. Both these studies furnish further evidence for the hypothesis suggested by McHenry (45) that thiamin is necessary for the synthesis of fat from carbohydrate.

Intraspinal effect.—In contrast with negative reports based on the thiochrome method (46, 47), the *Phycomyces* test demonstrates thia-

min (traces to 18.5 µg. per cent) in the cerebrospinal fluid. The values are unrelated to the vitamin content of the blood. Intraspinal injection of the vitamin was followed by rapid resorption into the blood from the lumbar and arachnoid spaces (48), thus indicating no advantage of this path over other routes of administration (48, 49) for the treatment of neural damage.

Thiamin and insulin.—Both insulin and thiamin are influential in carbohydrate metabolism, causing a lowering of blood pyruvic acid and of urinary phosphate (50). Awe (51) postulated that antineuritic effects may be ascribed to insulin and reported hydrolyzates of several insulin preparations to show a slight thiochrome reaction. Ahlström *et al.* (52) found on the contrary that thiamin is not present in pure crystalline insulin or its hydrolyzates, and Barnes & MacKay (53) reported that protamine zinc insulin is without effect upon the decreased appetite of rats suffering from thiamin deficiency.

Thiamin and acetylcholine.—Erspamer (54, 55) made the puzzling report that nonlethal doses of thiamin followed by nonlethal doses of acetylcholine caused death in rats, while lethal doses of thiamin, 220 mg. per kg., followed by lethal doses of acetylcholine, 300 to 450 mg., inhibited the toxic effects and retarded death. Polyneuritic pigeons also required much larger lethal doses of acetylcholine than did normal birds. However, studies of the heart action in thiamin avitaminosis in dogs and rats indicated that the deprived hearts were more resistant to certain drugs such as pitressin, epinephrine, and atropine than the controls, but were normal in reaction to acetylcholine (56).

Birkhäuser & Süllmann (57) found acetylcholine to be hydrolyzed more completely than acetylthiamin by various tissue extracts. There was no appreciable difference in the capacity of intact brain slices from polyneuritic and normal pigeons to synthesize acetylcholine (58). The interaction of thiamin with acetylcholine has been briefly reviewed by Bernheim (59).

Thiamin deficiency symptoms.—Dimick (60) differentiated between thiamin deficiency and deficiencies of other members of the vitamin-B complex in rats by the absence of the plateau period in the growth curve during thiamin avitaminosis. This clearly indicates the acute or crucial character of this deficiency. Swank (61) saw few or no morphological changes in acute cases of thiamin deficiency in pigeons but, in chronic cases, a Wallerian degeneration of both peripheral and spinal neurons which corresponded closely to the degree

of paralysis observed. The diet used was deficient in the B_2 vitamins, however, and these deficiencies were maintained long enough to produce the pathology found only in the chronic cases. Alexander (62) pointed out the identity of the vascular lesions produced by thiamin avitaminosis in pigeons with Wernicke's disease in man, the hemorrhagic polioencephalitis of chronic alcoholism.

Other thiamin-deficiency signs reported have been the following: diminution of both free and phosphorylated thiamin in the tissues of rats and pigeons (63) ; decreased blood sugar in hens (64) ; decreased glycogen in liver (65), muscle, and brain of rats (66) ; increased lactic acid of blood and muscles and increased pyruvic acid in brain, muscle, and blood of rats (67) ; and a rapid and progressive rise in the urinary bisulfite-binding substances (B.B.S.) in rats—as great as 900 per cent in advanced deficiencies (68). Isocaloric substitution of fat for sucrose in the diet diminished the excretion of B.B.S., but the values still remained at levels above the normal (68, 69). Robinson et $al.$ (70) studied the blood B.B.S. in thiamin deficiency and could not correlate its level with thiamin excretion.

De Soldati (71) found increased pulse rate and decreased blood pressure and, on autopsy, hydropic degeneration of the heart (72) in dogs with thiamin avitaminosis. Haynes & Weiss (73) are of the opinion that the accumulation of metabolites, e.g., pyruvic acid, is not the chief cause of the cardiac manifestations in thiamin deficiency in rats. MacDonald & McHenry (74) showed that the bradycardia of rats was cured specifically by thiamin and was not solely the result of inanition although it was not relieved by the administration of thiamin unless food was also given.

$Beriberi.$—Several papers have discussed the symptoms of classical beriberi (75, 76, 77, 78, 79) in relation to pure thiamin deficiency. The impression gained is that nerve and muscle degeneration and edema are probably ascribable to deficiencies of vitamin B_2, vitamin A, and protein.

$Assessment$ of $thiamin$ $status.$—Pannekoek-Westenburg & Van Veen (80) and Magyar (81) concluded from their studies of blood thiamin that proof of thiamin deficiency may not be drawn merely from the level of thiamin in the blood. However, Goodhart & Sinclair (82) found blood cocarboxylase to run closely parallel with the total amount of thiamin and thought it could be used with reasonable accuracy as an index of body saturation. In another study intravenous injection of 3 mg. of thiamin caused an increase of at least 10 per

cent in the blood thiamin in five to thirty minutes in normal subjects but only 2.8 per cent increase in cases of ulcer, carcinoma, goiter, and severe infection (83).

Thiamin excretion is also taken as an indication of the adequacy of intake. Normal subjects were found to excrete about 100 μg. in twenty-four hours (84, 85, 86), but in subjects suffering from thiamin deficiency the excretion was lower (86). Urinary excretion, after a test dose of thiamin, is a more reliable index of the degree of saturation. Normal subjects excreted 18 to 56 per cent of intravenously injected doses of 2 to 10 mg. within twenty-four hours (81, 87) while patients with thiamin deficiency excreted less than 18 per cent although the excretion increased after repeated injections (81, 86, 87). The extent of the deficiency may be measured by the number of test doses required for the excretion to rise to normal (88). Shorter periods of urine collection, three to four hours, immediately after the injection were thought to offer a more reliable index of deficiency (84, 89, 90). Thus 2.6 to 18 per cent of 1 mg. doses of the injected thiamin was recovered in the urine of normal subjects in three to four hours (84, 89).

Wang & Yudkin (91) suggested that an indication of the thiamin requirement may be obtained from the level of intake at which a rapid rise in excretion begins. When the daily intake of their three adult subjects reached 1.2 mg., there was a fairly sudden rise in output. The minimum percentage of the intake excreted occurred with daily intakes between 0.9 and 1.2 mg. which is in good agreement with the reputed requirement suggested by the League of Nations Commission (92). Only small decreases in thiamin excretion resulted from a large increase in fat or carbohydrate in the diet or from moderate exercise. This is in contrast with the finding of Guerrant & Dutcher (93) that in the rat exercise increased the thiamin requirement.

The human requirement.—Cowgill (94) placed the average adult requirement at 0.9 to 1.05 mg. per day or 30 μg. per 100 kcal., although there is considerable evidence that the optimum requirement is greater (about 1.5 to 2.0 mg. per day) (95, 96, 97). Preschool children need at least 70 to 75 μg. per 100 kcal. and Knott (98) has estimated 0.18 to 0.24 mg. as the minimum and 0.30 to 0.45 mg. per day as the optimum for infant needs.

Dietary levels.—Dietary surveys of recent years indicate that the average thiamin intake is very near the minimum and certainly not the optimum for human needs (99, 100, 101). A wide gap is said to exist between minimum and optimum requirements as indicated by

the beneficial effects of increasing the thiamin intake (102). Various hospital diets have been found to be suboptimal in thiamin content even though there may be an increased need in various disorders (103, 104).

Modern milling processes remove about 3/4 of the thiamin of rice (105) and 10/11 of that of wheat (94, 106). This decrease is serious when wheat furnishes 25 per cent or more of total calories, and refined sugar, completely lacking in thiamin, makes up another 10 to 20 per cent. No very satisfactory solution can be expected from a greater use of so-called protective foods, since more than half of the foods of an ordinary American diet contain no appreciable amount of thiamin (107) and, furthermore, considerable losses may result from canning and cooking.

Fortification of foods, particularly flour.—The need for adding thiamin to staple American foods has been emphasized by Cowgill (94). He suggested that addition of thiamin to cereals, especially to patent flour, would increase the thiamin intake of a larger part of the population than could be effected by any other single measure. The amount suggested was 225 µg. of thiamin per 100 kcal.

The British government recently decided to fortify white bread with thiamin and calcium (108). Contrary to gloomy predictions (109) the addition had no effect on appearance, palatability, and digestibility, and furthermore, due to the acidity of flour, thiamin was found to resist destruction during doughing and baking (110). Bailey (111) also discussed addition of crystalline thiamin to white bread, the use of concentrates rich in thiamin, and the use of high-vitamin (thiamin-enriched) yeast. Chick (112) demonstrated with rats that the growth-promoting value of whole wheat flour is considerably superior to that of white flour even when the latter is enriched with protein, minerals, and thiamin. This inferiority is attributed to the lack of the B_2 vitamins.

The question still unsettled is this: Is it better to make partial restoration to white flour of the nutrients lost in milling or to wait until full restoration becomes possible? No commercial products yet available in sufficient amount, such as high-vitamin yeasts or synthetic vitamins, accomplish such complete restoration.

Clinical uses of thiamin.—Thiamin deficiency induced in human subjects by an intake restricted to 150 µg. per day produced a condition resembling neurasthenia (113). Fantus (114) has reviewed clinical thiamin subvitaminosis, indicating the various obvious factors

influencing it as well as the more subtle ones. Alcoholism (114 to 118), pregnancy (114, 119, 120), and gastrointestinal disturbances (114, 121) are frequent predisposing factors in the causation of thiamin deficiency. Thiamin administration caused a high incidence of improvement in polyneuritis due to various causes (122, 123, 124, 125), and disappearance of neuropathic symptoms of pellagrins (126, 127) and of the symmetrical peripheral neuropathy in some diabetics (128).

The development of typical pellagra symptoms has been observed during the treatment of patients with high doses of thiamin (129, 130), an observation which supports the view that vitamin-B therapy should be polyvalent to prevent disturbance of the B-complex balance and the development of some form of B-avitaminosis.

Study of patients with nerve deafness by Selfridge (131) showed that in nearly all cases, the urinary output of thiamin was below normal. Thiamin was reported to cause complete relief of tinnitus and improvement in hearing (132), although Childrey (133) reported no success in patients with impaired hearing.

Thiamin has been used successfully in the treatment of the neuralgia of varicose ulcer (134), in cases of herpes zoster (135), and in irradiation sickness (136). Beneficial effect was reported in cases of multiple sclerosis (137), major trigeminal neuralgia (138), infant acrodynia (139, 140), dysphagia (141), and, when given in conjunction with suprarenal cortex, in hyperemisis gravidarum (142). No favorable action of thiamin was seen in cases of postdiphtherial neuritis (143) and schizophrenia (144). Thiamin deficiency may be associated with the narcosis of encephalitis and the physiology of sleep (145) and with spastic colitis (146), but is not related to the disturbances in carbohydrate metabolism of true diabetes mellitus (147). Most of these conditions which were once thought to have been benefited by thiamin therapy are obviously due to either neural or gastrointestinal derangements, conditions which can no longer be regarded as specifically characteristic of thiamin deficiency.

RIBOFLAVIN

Some of the outstanding events of 1940 in the field of riboflavin research were (a) the discovery of early ocular lesions in human cases of uncomplicated ariboflavinosis, (b) improvements in methods of

assay, and (c) the indication that cheilosis, thought in 1939 to be specific to ariboflavinosis, is curable by pyridoxin.

Methods of determination.—Four methods are employed for the determination of riboflavin: the photometric, the fluorometric, the microbiological, and the biological. Improvements on each of these have been suggested and assays of natural products by old and new methods reported.

Schumacher & Heuser (148), using yeast, compared the photometric method with assays on the chick, hen, and rat and found good agreement between these methods. However, Kemmerer (149) reported dissatisfaction with the colorimetric method and recommends further study of the fluorometric and microbiological methods. Ferrebee (150) suggested a fluorometric method to be used in determination of urinary uroflavin and Hodson & Norris (151) described a fluorometric method for foodstuffs, the results of which agreed well with those of photometric and microbiological assays. Feeney & Strong (152) found that the microbiological method measured both free and combined forms of riboflavin. Fraser *et al.* (153) suggested that the bacterial method of Snell & Strong (154) may be useful in determining the nutritional status of dogs and rats with respect to riboflavin because the depletion can be observed in the urine before the animals manifest significant symptoms of deficiency. Some difficulty has been experienced with the biological assay in obtaining a basal diet supplemented by adequate amounts of the other B_2 vitamins yet free from riboflavin. In three methods now proposed (155, 156, 157), the essential difference is in the source and treatment of the material which is used as a source of the vitamin-B_2 complex, that is pyridoxin, pantothenic acid, and unknowns. Henry *et al.* (158) compared the method of El Sadr (155) with a fluorometric method in the assay of milk and reported good agreement when the milk was fed at levels up to 10 μg. daily. Wagner *et al.* (156) applied their method to the assay of such materials as yeast, liver extract, and grass.

Distribution.—Fixsen & Roscoe (159) have summarized much of the work on the distribution of riboflavin in their table on the vitamin content of human and animal foods. Kunerth & Riddell (160), in a study of the effect of the stage of lactation on cow's milk, reported that there was little change in the riboflavin content of milk after the first month of lactation. The average milk contained 2 mg. of riboflavin per liter. Other studies cover the riboflavin content of aquatic

products (161), liver (162, 163), and some common fruits and vegetables (164). The green vegetables such as spinach, lettuce, and broccoli prove to be the best vegetable sources of riboflavin, although little difference is found between the inner and outer leaves of lettuce (165). Hunt & Bethke (166) found that alfalfa plants exposed to strong sunshine for forty-eight hours after cutting suffered a loss of about 25 per cent of their riboflavin content. That the amount of riboflavin in the diet influences the amount which can be stored in the tissues was substantiated by Hodson (167) in his study of the vitamin in chicken tissue. Yamasaki (168) isolated a pure flavin, identical with riboflavin, from rice which had been fermented.

Physiological and pathological role.—As the yellow enzyme, of which riboflavin is a constituent, is concerned with carbohydrate metabolism, riboflavin may well be of importance in muscular effort. Thus, Minibeck & Verzar (169) found that the riboflavin content of the livers of rats on a diet lacking in riboflavin decreased much more rapidly when the rats were forced to take exercise. Yudkin & Geer (170) reported that massive doses of riboflavin had no protective action against the development of galactose cataract in rats even though riboflavin deficiency is known to cause cataract formation in rats. Axelrod et al. (171) in uncomplicated riboflavin deficiency in the dog, noted loss of weight with collapse and death in four to six weeks. Hughes (172) found the minimum daily requirement of riboflavin for the young growing pig to be between 1 and 3 mg. per 100 pounds.

Engel et al. (173), producing riboflavin deficiency in the hen, found not only lowered hatchability of eggs, but also myelin sheath degeneration in the sciatic nerve in 60 per cent of the surviving embryos. Kahler & Davis (174) reported low riboflavin in the livers of rats in which tumors had developed although the muscle values indicated no real deficiency.

Coenzyme I and riboflavin in the livers of rats fed dimethylaminoazobenzene (butter yellow) were reported (175) to be decreased decidedly although the Q_{O_2} was not affected. These rats develop liver cancer but appear to be protected by maintenance of liver flavin and coenzyme through administration of dried brewer's yeast.

Ocular lesions in ariboflavinosis.—Sydenstricker et al. (176, 177) reported severe interstitial keratitis and other eye abnormalities in forty-seven patients suffering from riboflavin deficiency, with rapid improvement when riboflavin therapy was instituted. Other specific

symptoms were a peculiar glossitis, seborrheic dermatitis, and cheilosis. The corneal keratitis of syphilis yielded also to riboflavin treatment. These important findings of keratitis in human beings followed that of Bessey & Wolbach (178) of corneal vascularization as the first symptom of ariboflavinosis in rats. The twilight blindness reported by Pock-Steen (179) in 109 cases of leiodystonia and sprue was alleviated in 78 cases by riboflavin but not by vitamin A as was found also in the series reported by Kruse et al. (177). Apparently there is much similarity between the conjunctival and corneal lesions resulting from riboflavin and vitamin-A avitaminoses. The singularly uncomplicated nature of these ariboflavinoses is unique, as is also the early appearance of the ocular lesions.

Quite similar was the experience of Johnson & Eckardt (180) who treated thirty-six patients with rosacea keratitis with 3 to 4.5 mg. riboflavin and saw prompt healing of the corneal lesions in thirty-two cases. Only two of the thirty-six patients had a history of adequate riboflavin intake and these two did not improve with riboflavin administration. They ascribed the rosacea keratitis to riboflavin deficiency. One case of pemphigus vulgaris was also reported cured by riboflavin (181).

Riboflavin complexes.—Riboflavin has been found in biological materials as flavin nucleoside, flavin mononucleotide, and flavin adenine dinucleotide. The latter is an important co-oxidase. Riboflavin is essential for the formation of flavin adenine dinucleotide. Klein & Kohn (182) examined the flavin adenine dinucleotide content of human blood cells both *in vitro* and *in vivo* and reported an increase of the flavin adenine dinucleotide content after addition or ingestion of riboflavin. Likewise this complex was increased in the liver and heart after the injection of lactoflavin into deficient animals (183). Subrahmanyan et al. (184), after purification of a liver flavoprotein which catalyzed the oxidation of aldehydes, found that the preparation contained 0.17 per cent riboflavin phosphate, present as alloxazine adenine dinucleotide.

The discussion initiated by Laszt & Verzar (185) as to whether riboflavin can be phosphorylated by the adrenalectomized rat for production of the yellow respiratory enzyme has continued. Experiments by both Bruce & Wien (186) and Nelson (187) indicated that neither riboflavin nor riboflavin phosphoric acid had any effect on growth or survival time of adrenalectomized rats in the absence of replacement therapy. Ferrebee (188), using the fluorescent method

which he had previously used on urine, found that the phosphoryla-
tion of riboflavin and also of thiamin was essentially normal in
adrenalectomized rats. This attractive theory of Verzar and co-
workers apparently has received no experimental support.

PYRIDOXIN

The outstanding events in the field of pyridoxin research in 1940
were: (a) the interrelation of fatty acid and pyridoxin deficiency in
rat acrodynia (204). (b) The cure of cheilosis by pyridoxin (218).
(c) The development of a chemical method for determination of
pyridoxin (192). (d) The alleviation of muscular dystrophy and
Parkinson's syndrome in humans by intravenous injection of syn-
thetic pyridoxin (225, 226).

Chemical estimation.—Scudi *et al.* (192) made use of Gibb's
phenolindophenol reaction for the estimation of pyridoxin. During
the development of the method, Scudi *et al.* (193) noted that the re-
action of pyridoxin with indophenol does not occur in the presence of
a borate buffer. Pyridoxin was found to undergo combination with
boric acid; the latter, with a coordination number of 4, is linked to
two molecules of the vitamin through oxygen atoms. This complex
has the physiological activity of pyridoxin.

Swaminathan (194) made use of the diazo reaction and phenol
reagent which reacts with pyridoxin to give an azo color which can
be read colorimetrically. The method is said to be applicable to all
foods and estimates as little as 10 μg.

Physiology.—Fouts *et al.* (195) and Borson & Mettier (196)
offered further confirmation that the hypochromic microcytic anemia
produced in dogs by a diet deficient in pyridoxin could be cured by
crystalline and synthetic pyridoxin. Döllken (197) found that prep-
arations of pyridoxin from liver hastened the regeneration of hemo-
globin and erythrocytes in the blood of rabbits made anemic by vene-
section.

Rat curative and prophylactic tests indicated that natural and
synthetic pyridoxin were identical in their physiological actions (198).
The di- and triacetyl derivatives were as potent as pyridoxin itself
on an equimolecular basis (199). Pyridoxin cured the florid der-
matitis (200, 201) as well as "ringtailed" condition (202) in rats
deprived of this vitamin. In prolonged pyridoxin deficiency rats de-
veloped epileptiform fits which were prevented and cured by pure

pyridoxin (200, 203). Similar fits had been seen previously in swine and dogs.

Schneider *et al.* (204) showed that acrodynia produced in rats by a diet free from fatty acids and all water-soluble vitamins except thiamin and riboflavin can be cured by "essential fatty acids" and that this action is independent of pyridoxin. It can also be cured by rice bran concentrate and this action is independent of the fatty acids, but is dependent upon pyridoxin plus a second "accessory factor" present in the filtrate from fuller's earth treatment. Pyridoxin in crude or crystalline form had only a temporary effect on acrodynia in the absence of the second factor. The Burr & Burr (205) syndrome which appeared to differ only in its severity from true acrodynia could be produced by the addition of 1 to 8 per cent of dried brewer's yeast to the acrodynia-producing diet of Schneider *et al.* (204) and was cured both by the essential fatty acids and rice bran concentrate. No comment is made in this report as to the effect on the growth of rats which had been maintained on the acrodynia-producing diet and which were cured by fatty acids or the vitamins. This is a puzzling problem, for obviously at least three deficiencies are involved: essential fatty acids, pyridoxin, pantothenic acid and, in addition, any other as yet undefined filtrate factors required by the rat. Yet restoration of one of these missing factors, the fatty acids, cured the syndrome. If the other factors, pyridoxin and filtrate factors, were supplied together a cure was also effected. This equivalence of widely different nutrilites is unique in rat nutrition and requires further study. Tange (206) and Salmon (207) also came to the conclusion that the presence of both pyridoxin and essential fatty acids is necessary for the prevention of dermatitis in rats. Supplee, Bender & Kahlenberg (208) advanced the theory that rat acrodynia developed by a pyridoxin-deficient diet cannot be cured or prevented by pyridoxin unless another complementing factor is supplied. This is essentially the "filtrate factor" of numerous other investigators.

Gavin & McHenry (209) reported that pyridoxin, given in conjunction with thiamin, riboflavin, and choline, to rats fed a fat-free diet caused a slight increase in body fat and an increase in body weight. Pyridoxin did not prevent the deposition of fat in the liver which resulted when thiamin was given to depleted animals. The amount of liver fat was normal if choline was administered either alone or in combination with pyridoxin, nicotinic acid, or riboflavin but no supplementing effect by pyridoxin could be detected.

Emerson & Evans (210) reported cessation of growth of male rats placed on a pyridoxin-deficient diet at about the 100th day, and grave failure of sexual development by the 150th day.

Covey & Forbes (211) reported that about 60 per cent of the total pyridoxin in aqueous hog liver extract was in a conjugated form insoluble in 80 per cent alcohol.

Toxicology.—Unna & co-workers (212, 213) reported that subcutaneous doses of 3.1 gm. of pyridoxin per kg. body weight and oral doses of 4 gm. per kg. were lethal for rats. Doses up to 1 gm. per kg. had no untoward effects, but higher doses produced tonic convulsions suggestive of nervous system involvement. Daily feeding of 10 mg. per kg. for three months in rats, dogs, and monkeys had no effect on weight, hemoglobin, erythrocytes, leucocytes, or blood counts; and 20 mg. per kg. given intravenously to cats had no effect on blood pressure or respiration. In concentrations of 1 part in 2,000, pyridoxin hydrochloride stimulated contractions of isolated intestine and uterus (201), but in concentrations of 1 part in 10,000, it had no effect (212). A curious autopsy finding in the rats killed by excessive doses was enlargement of the adrenals with massive hemorrhages into the cortex (213). This is similar to the condition found in pantothenic acid deficiency (214) and brings forward again the possible antagonism of these two vitamins.

Excretion.—Scudi et al. (215, 216) estimated colorimetrically the pyridoxin in urine and found that in fasting dogs, the excretion of pyridoxin is essentially complete six hours after oral administration; the recovery was only 20 per cent. In nonfasting human subjects, 8.7 to 7.9 per cent (216, 217) of the intravenous dose of 50 mg. was recovered in one hour, and 7.6 per cent of the oral dose of 100 mg. was recovered in four hours, although excretion appeared to be practically complete after these times. Spies et al. (217) found that nine patients with various B-complex deficiencies excreted an average of 0.5 per cent of the test dose of pyridoxin while four patients suspected of pyridoxin deficiency excreted an average of 0.2 per cent.

Clinical use of pyridoxin.—Smith & Martin (218) reported successful treatment of cheilosis associated with pellagra, sprue, celiac disease, and digestive upset with intravenous doses of 20 to 50 mg. pyridoxin daily. Healing of the fissures at the corners of the mouth resulted in two to five days. In one case, healing took place on a diet lacking in riboflavin and in another, cheilosis occurred on a diet rich

in riboflavin; therefore, the authors question the contention of Sebrell & Butler (219) that cheilosis is specifically due to riboflavin deficiency.

Vilter *et al.* (220) reported that 50 to 100 mg. pyridoxin given intravenously daily to pellagrins with macrocytic anemia and to two pernicious anemia patients caused clinical improvement within forty-eight hours and later a slight reticulocytosis, never above 5 per cent.

Pyridoxin has been reported to cause notable improvement in cases of pseudohypertrophic muscular dystrophy (221), and when given in conjunction with α-tocopherol, to cause partial remission in the symptoms of arsenic peripheral neuritis (222). Pyridoxin is of doubtful value in infantile seborrhea (223) and of no value in anemia associated with alcoholic or endemic pellagra (224). However Jolliffe (225) and Spies *et al.* (226) have reported relief of symptoms in paralysis agitans patients by intravenous injection of 50 to 100 mg. pyridoxin. Apparently some of these patients have pyridoxin deficiencies.

Role of pyridoxin in lower forms of life.—Pyridoxin has been shown to be an accessory growth factor for mosquito larvae (227), yeast, *Saccharomyces cerevisiae* (228), *Streptococcus zymogenes* (229), *Streptococcus hemolyticus* (230), and *Staphylococcus albus* (231). It apparently functions as an activator for root formation in plant cuttings (232).

Nicotinic Acid and Nicotinamide

Methods of determination.—All the recently studied chemical methods for the determination of nicotinic acid are based on the yellow color produced when the pyridine nucleus reacts with an aromatic amine in the presence of cyanogen bromide. The methods vary as to the amine used in the reaction and as to the procedure used to make the biological material adaptable to colorimetric measurement. Harris & Raymond (233) in a method proposed for determinations on urine used *p*-aminoacetophenone, the solutions to be protected from the light, and the nicotinic acid content of the sample to be estimated by extrapolation of values obtained from added known amounts of nicotinic acid. Kodicek (234) extended this method to other biological materials. Melnick & Field (235) adapted their method, using aniline as the amine, to use with the Evelyn photo-

electric colorimeter. They used direct acid hydrolysis followed by preferential charcoal adsorption of the colored compounds in the hydrolyzate. They (236) rejected Harris & Raymond's (233) use of aniline in the blank, on the ground that side reactions occur therein which are not present in the cyanogen bromide reaction mixture. Melnick, Robinson & Field (237) showed that by prolonged acid hydrolysis followed by alkaline hydrolysis, nicotinuric acid and trigonelline can be differentiated from nicotinic acid in urine. Trigonelline, susceptible to alkaline but not acid hydrolysis, appeared to be a normal urinary constituent, markedly increased by coffee drinking. Both trigonelline and nicotinuric acid have been isolated from urine following administration of nicotinic acid, but trigonelline, which is widely distributed in nature, has been found to exert no antiblacktongue effect. Nicotinuric acid apparently has antiblacktongue value. Smoking was found to increase urinary trigonelline in some cases and in others to cause excretion of free nicotine. No antipellagra value has been found in trigonelline or pyridine but equally curative are nicotinic acid and nicotinamide, and somewhat less so is nicotinuric acid. Trigonelline, but not nicotinuric acid, was found to be a normal constituent of urine (238). When 500 mg. of nicotinic acid were given after a meal a rapid increase in "nicotinic acid" excretion took place, 51 per cent as trigonelline, 36 per cent as nicotinuric acid, and 13 per cent as nicotinic acid or amide. Greater excretion followed the test dose if taken by a fasting subject. Nicotinamide produced lesser and slower excretion but gave a higher percentage of trigonelline. Prompt and continuous change of both substances to trigonelline and nicotinuric acid evidently occurred.

Rosenblum & Jolliffe (239) raised the objection that these methods for nicotinic acid determination are too tedious and time consuming to be used in routine clinical analyses and have modified that of Bandier & Hald (240) so as to be applicable to the rapid determination of nicotinic acid and nicotinamide in urine. However, trigonelline, apparently an end product of nicotinic acid metabolism, is not included by this method. Perlzweig, Levy & Sarett (241), being unable to obtain reproducible and concordant results with previous methods, devised a new colorimetric method for the determination of nicotinic acid in urine. To differentiate trigonelline from nicotinuric acid and nicotinamide they follow essentially the same technique as that of Melnick *et al.* (238). Only 10 to 25 per cent of 100 or 200 mg. doses of nicotinic acid could be found in the urine as trigonelline,

nicotinuric acid, and nicotinic acid. Schindel (242), in commenting upon the methods that use dinitrochlorobenzene, pointed out that the creatinine of urine yields a yellow color with this reagent. As yet no simultaneous comparative results using two or more of these chemical methods upon the same materials have been published and until this is done, no estimate can be made of the reliability of the various methods.

Nicotinic acid in blood.—Kodicek (243), using the technique of Harris & Raymond, assayed animal tissues, blood, and some foods. The results compared favorably with the reputed pellagra-preventing values of the various foods. In horse and sheep blood about 0.47 mg. per cent nicotinic acid was found, all of it in the red cells. Melnick *et al.* (244) found average values for blood nicotinic acid in humans to be 0.62 mg. per cent for females and 0.69 mg. per cent for males, 90 per cent of it in the corpuscles. Neither a recently ingested meal, coffee drinking, nor smoking appreciably affected the blood nicotinic acid values. Villella (245) reported 0.36 mg. per cent for the average blood nicotinic acid level of both sick and healthy subjects whereas Querido *et al.* (246) found the average to be 0.73 mg. per cent. Probably differences in the specificity of the methods of determining nicotinic acid account in part for these variations.

In foods.—Waisman *et al.* (247) have determined the nicotinic acid potency of numerous food materials and chemical compounds by use of "blacktongue" dogs maintained on the modified Goldberger diet. Aykroyd & Swaminathan (248) assayed many cereals and cereal products by the aniline–cyanogen bromide method. Aykroyd (249) found that raw rice lost about two-thirds of its nicotinic acid in the milling process, but if it were parboiled before milling, the loss was reduced due to the diffusion of some of the nicotinic acid into the endosperm. Kringstad & Thoresen (250) reported on the amount of nicotinic acid in fish and fish products.

Nicotinic acid and coenzymes I and II.—It is well established apparently that in man, dogs, monkeys, and pigs, nicotinic acid is a vitamin with a set of deficiency symptoms which are cured by administration of this substance or of such conjugated forms as will yield it in the animal body. Likewise it is established that nicotinamide is part of the molecules of two coenzymes which play an important part in intracellular oxidation. These are coenzyme I (diphosphopyridine nucleotide), and coenzyme II (triphosphopyridine nucleotide). Whether the vitamin character of nicotinic acid is solely

due to the need of it for production of these coenzymes is a question of interest. In spite of the efforts of a number of investigators it is still only partially answered. The quantities of coenzyme in blood and urine appear little affected by nicotinic acid ingestion or deficiency but in certain tissues, particularly liver and muscle, the content rises and falls with nicotinic acid intake. Some function other than coenzyme production may be performed by this vitamin.

Pittman & Fraser (251), by the use of *Hemophilus parainfluenza* determined that the excretion of V factor (coenzyme I, coenzyme II, and possibly unknown related compounds) in the urine of dogs is not influenced by a diet either deficient or rich in nicotinic acid. However, the amount of V factor in the liver, muscle, and heart of dogs suffering from blacktongue was appreciably less than in corresponding tissues of normal dogs. These workers reported no difference in the levels of factor V in the blood between the two groups of animals. Axelrod *et al* (252), employing the specific yeast fermentation method for the estimation of coenzyme I, found that the ingestion of large amounts of nicotinic acid by human subjects increased the coenzyme I level of the blood and that this level varied with the amount of nicotinic acid fed. It was suggested that nicotinic acid probably diffuses into the red blood cells from the plasma and is there utilized for the synthesis of the pyridine nucleotides. Kohn (253) verified his method by the addition to blood of known amounts of coenzymes and also noted that they are found only in the corpuscles.

Synthesis by the rat.—Dann & Kohn (254) using the factor V method of Kohn (255) on tissues of rats fed low levels of nicotinic acid found that the coenzyme content of the liver, kidney, and thigh muscles of rats was decreased only about 10 per cent by deprivation of nicotinic acid. Since, on one of the deficient diets, the growing rats synthesized coenzymes at a rate that utilized nicotinic acid more rapidly than it was ingested, they suggested that nicotinic acid itself was being synthesized by the rats. Shourie & Swaminathan (256) also observed that the rat must be able to synthesize nicotinic acid, for although the animals receiving this substance excreted increased amounts in the urine, no significant differences were found in the nicotinic acid contents of the tissues of animals which received, or which were deprived of, nicotinic acid.

Value of other pyridine or pyrazine compounds.—In an attempt to identify pyridine derivatives that might replace nicotinic acid in

some of its functions, Dann *et al.* (257) found that neither quinolinic acid, pyrazine monocarboxylic acid, nor pyrazine 2,3-dicarboxylic acid was effective in raising the V-factor content of blood cells when these acids were taken orally by humans or when incubated under sterile conditions with defibrinated human blood. None was effective in curative trials on blacktongue in dogs, but 20 mg. daily of quinolinic acid was preventive over a period of 156 days. This may be contrasted with 0.15 mg., the minimum protective daily dose of nicotinic acid. These results conflict with the earlier report of Vilter & Spies (258) that pellagrins were cured by quinolinic acid and with those of Bills, McDonald & Spies (259) on the antipellagra effectiveness of the pyrazine acids. The method of determining V factor was different in the 1940 study from that of the 1939 reports but nevertheless a re-examination of the antipellagra effect of these acids seems indicated before a species difference between dogs and humans in utilization of nicotinic acid analogues is considered established.

Smith *et al.* (260) reported that 29 mg. per kg. of nicotinic acid diethylamide (nikethamide) was effective in curing ten out of fourteen dogs of blacktongue, and 72.5 mg. per kg. was required to cure all of the dogs (as contrasted with 5 mg. nicotinic acid ker kg.). These workers report no flushing of the skin in human subjects following administration of nikethamide. Boyd & Forde (261) observed visible excitement in rats, guinea pigs, cats, rabbits, and pigeons with a marked increase in the output of total urinary, fecal, and residual water when doses of nikethamide of 10 to 25 mg. per 100 gm. of body weight were given.

The flushing of the skin produced by nicotinic acid.—Nicotinic acid and its sodium, ethyl, and monoethanolamine salts produce vasodilation of the skin and increase in skin temperature when given intravenously in doses of 20 mg. No vasodilation followed administration of comparable amounts of quinolinic acid, nicotinamide, nicotinic acid N-diethylamide, or pyrazine 2,3-dicarboxylic acid (262). Popkin (263) noted transitory flushing of skin, tingling, itching, and sensations of heat when nicotinic acid was administered orally but Field & Robinson (264) observed that oral doses up to 500 mg. caused none of the flushing or other symptoms sometimes reported. Abramson *et al.* (265) studied the peripheral vascular effects of nicotinic acid by means of the plethysmographic method; they found no definite or consistent alteration in blood pressure or pulse rate, and suggested

that the flushing effect is due to local changes in blood vessels rather than to an increase in cardiac output.

Deficiencies in farm animals.—Davis *et al.* (266) have suggested that the necrotic enteritis of pigs is not a primary infection but is a secondary complication resulting from invasion of the intestinal wall by commonly occurring microorganisms after a deficiency of nicotinic acid has developed.

The synthesis of nicotinic acid by sheep has been reported by Winegar, Pearson & Schmidt (267). After lambs had been on a diet deficient in nicotinic acid for eight months, nicotinic acid was excreted at essentially the same level as by those on the control diet and the addition of nicotinic acid to the deficient diet somewhat irregularly increased the excretion in the urine. Subsequent work (268) showed that there was no difference between the nicotinic acid level of the blood or in its excretion in the case of lambs on diets low in nicotinic acid and of those on stock diets. However this same characteristic has been observed in other species which do develop nicotinic acid deficiency (251, 269). Determination of the nicotinic acid or coenzyme content of tissues appears to be a more reliable criterion.

Clinical uses.—Nicotinic acid is important in the nutrition of lower organisms. It is essential in the growth of dysentery bacilli (270, 271) and of *Proteus morganii* (272). Vilter *et al.* (273) reported low concentrations of coenzymes I and II in the blood of patients with acute lobar pneumonia and a return to normal in twenty-four to forty-eight hours after the crisis had been induced. A similarly normal range of values was seen by Kohn *et al.* (269) in several pneumonia cases after the crisis.

Relation to pellagra.—The relation of nicotinic acid to pellagra has been well reviewed by Elvehjem (274, 275). The conclusion appears to be that the pellagra caused by nicotinic acid deficiency can be cured by nicotinic acid therapy, but that multiple deficiency pellagra can only be cured by supplying all of the lacking vitamins. Numerous cases of pellagra have been reported (276, 277, 278, 279). Musick (280) found in fifty cases that the curative effect obtained with whole yeast was no better than that with nicotinic acid alone. Salvesen (281) suggested that overdosing with vitamins B_1 and C, which he supposed to act synergetically, may bring about a relative insufficiency of nicotinic acid.

Nicotinic acid therapy has become important in treatment of many other diseases, many of which are probably subacute instances of the

pellagra caused by nicotinic acid deficiency. Goodall (282) has obtained improvement following such therapy in cases of sore tongue, anorexia, stomatitis, diarrhea, mental dullness, and infected scabies.

Nicotinic acid in conjunction with thiamin chloride has proved beneficial in cases of Ménière's syndrome and multiple sclerosis (283, 284). The incidence of nausea, headache, and cyanosis concomitant with sulfanilamide or sulfapyridine administration has been greatly decreased by simultaneous dosage with nicotinic acid. Likewise the disagreeable reactions following high voltage roentgen therapy have been alleviated with this medicament (285, 286). Conditions of xerostomia, summer diarrhea in infants, encephalopathic syndrome, and Vincent's disease, have been successfully treated with nicotinic acid (287, 288, 289, 290). Siedek & Reuss (291) have reported that with nicotinamide treatment there was improvement in fat absorption in a patient suffering from pellagra and deficient fat absorption. The treatment of peripheral vascular diseases with nicotinic acid has been discouraged by Popkin (263) and by Bean & Spies (262) because however favorable may be its effects on the peripheral circulation, they are of short duration.

PANTOTHENIC ACID

Chemistry of pantothenic acid.—Perhaps the most outstanding event of the year 1940 in the vitamin field was the identification and synthesis of pantothenic acid. The existence of the substance was first noted in 1931 (292) as a nutrilite necessary for the growth of a certain strain of yeast. In 1933 (293) the name pantothenic acid was suggested because of its apparently universal occurrence in living matter. The isolation of the factor proved to be difficult due to its low concentration in natural sources, its hygroscopic property and extreme solubility in water, its instability in both acid and alkali, and its lack of easily recognized characteristics such as color, odor, and reducing properties.

Nevertheless, highly active concentrates of the factor were prepared and certain properties determined. Williams & Rohrmann (294), having found that the β-amino acid, aspartic acid, promoted yeast growth, were fortunate in finding that β-alanine was also active. They then looked for and found β-alanine in the cleavage products of their pantothenic acid concentrates. A search then began for the

identity of the acid fragment of the molecule. The nearly pure calcium pantothenate was found to yield a hydroxy acid lactone as well as β-alanine and the molecule was therefore assumed to be a peptide-like compound (295). Certain properties of the acid fraction were determined in the nearly pure product made from natural sources (296). The acid fraction was shown to be an α-hydroxy-γ-lactone, with no β-hydroxy group. No adjacent hydroxyl groups were present in the pantothenic acid molecule. The identity of the lactone cleavage product was established by Stiller *et al.* (297) as α-hydroxy-β, β-dimethyl-butyrolactone, a compound which had been known for many years. Snell, Strong & Peterson (298) as well as Williams and associates (299) had earlier coupled β-alanine with the impure and unidentified hydroxy acid obtained from the pantothenic acid concentrates and had shown the product to be biologically active. Woolley *et al.* (300) had also recognized the lactone-forming character of the hydroxy acid and suggested the position of the hydroxyl groups. After the correct structure of the lactone fragment had been established (297) several methods of coupling it with β-alanine were described (301, 302, 303, 304). The synthetic product

$$[HOCH_2 \cdot C(CH_3)_2 \cdot CHOH \cdot CONH \cdot CH_2 \cdot CH_2 \cdot COOH]$$
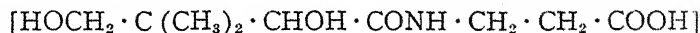
was found to be identical with the corresponding substance obtained from natural sources (301). Of the two optical enantiomorphs produced by synthesis, only the natural dextrorotatory form proved to be biologically active. Most of the tests for biological activity made by those engaged in identification of the lactone (301) involved the use of bacteria, but similar results were obtained with rats and chicks (301, 302).

Several derivatives or analogues of pantothenic acid have been found biologically inactive, viz. the diphosphate, like all other esters tried (305), and four amino acid analogues (306). Synthetic hydroxy-pantothenic acid, however, was found to possess striking but variable biological activity (by bacteria growth tests only) (307). Since natural concentrates and synthetic pantothenic acid gave concordant results, it was concluded that natural pantothenic acid is a single specific substance and that hydroxypantothenic acid probably does not occur in the natural concentrates. Hydroxypantothenic acid was later shown to have some growth-promoting value for rats fed diets

deficient in pantothenic acid (308). The toxicity of synthetic pantothenic acid was found to be low (309).

Pantothenic acid in the nutrition of chicks.—The identity of pantothenic acid with the chick antidermatitis factor was apparently satisfactorily established even before synthetic pantothenic acid became available.

The requirement of chicks was established by Jukes (310) as 1.4 mg. per 100 gm. of diet by means of a highly potent preparation of the natural product. Synthetic *dl*-pantothenic acid was found by chick assay (302) to have one half the potency of the natural substance, thus confirming the finding that only the natural isomer is biologically effective.

Snell *et al.* (311) by both yeast and lactic acid bacterial assay (312) established the low (30 to 70 per cent of normal) pantothenic acid levels in liver, kidney, muscle, blood, brain, and cord of chicks which had been fed diets deficient in pantothenic acid. This is further proof of the vitamin character of pantothenic acid for this species.

Dimick & Lepp (313) confirmed the finding (314) that another factor adsorbed from rice bran concentrate is required by chicks along with pantothenic acid when a heated diet is used. They found synthetic pantothenic acid also inadequate for protection of the rat from filtrate factor deficiency, but that it was supplemented perfectly by a nonadsorbable factor in the rice bran concentrate. Thus they postulate two new additional factors in the B complex.

Earlier work has shown the need of pantothenic acid in the hen's diet for hatchability of eggs and for maintenance of normal structures of the spinal cord in the chick (315) but nothing else has developed as to the role of pantothenic acid in avian physiology.

Pantothenic acid and the anti-grey hair factor.—It was early assumed that pantothenic acid is needed by the rat both for growth and for maintenance of normal pigmentation of the fur. The "rat growth filtrate factor" was clearly shown in several laboratories in 1939 to contain pantothenic acid, but doubt has persisted as to the presence of one or more other filtrate constituents needed by this species. Evidence has been offered for the existence of a separate factor variously named "anti-achromotrichia factor," "anti-grey hair vitamin," "vitamin B_x," "factor W." Woolley (316) pointed out the multiple nature of the rat filtrate factor and stated that after sufficient depletion, young rats did not respond with growth when even large doses of pantothenic acid were given, although liver extracts evoked

excellent response. Biotin appeared to be an insufficient addition to the pantothenate. Black, Frost & Elvehjem (317) reviewed the "factor W" situation and came to the conclusion that their original basal diet deficient in "factor W" was also deficient in both pyridoxin and pantothenic acid, but found that a growth factor still remained in the residue of liver extract freed of the filtrate fraction which was required by the rat and for which they retained the designation factor W. György, Poling & Subbarow (318) claimed a cure of greying of hair in rats by 75 to 200 µg. pantothenic acid daily but added that this is not the only factor concerned in the cure of nutritional achromotrichia. Later, however, György & Poling (319) reported a practically complete cure of greying in five to seven weeks by daily doses of 75 to 100 µg. Still later, they (320) reported that pantothenic acid cures of the greyness in rats and mice were not complete unless biotin (321) was also given (0.25 µg. daily of crystalline biotin methyl ester). The basal diet contained 10 per cent dried heated egg white. Even with the biotin added to the pantothenic acid the fur pigmentation was not entirely normal. Another supplementation of pantothenic acid by a biotin-like preparation was reported by Hegsted *et al.* (322) for the cure of a new dermatitis seen in chicks receiving an adequate amount of pantothenic acid in a purified diet.

Earlier in the year Robbins & Hamner (323), using a filtrate factor concentrate made from yeast and known to be rich in both pantothenic acid and the anti-grey hair factor, demonstrated its potency for growth stimulation of *Phycomyces* either alone or along with the Z_2 factor prepared from potato extract. They believed that the yeast concentrate contained both of their factors Z_1 and Z_2 shown to be needed by *Phycomyces,* but that it was particularly rich in Z_1, which appeared to be identical with biotin. Pantothenic acid had been reported ineffective for *Phycomyces* (324).

Dimick & Lepp (313) suggested that in addition to pantothenic acid another new factor is required by rats deficient in filtrate factor to produce complete cure of the greying of hair. They found the new factor in the filtrate from rice bran concentrate treated with fuller's earth, and further treated with charcoal at pH 4 to 5; only 14 µg. per cc. of pantothenic acid remained.

Mohammad *et al.* (325) concluded that two factors are present in the rat filtrate factor complex, one essential for growth, the other for normal color of the pelage. Both factors were extracted by acid-ether from brewer's yeast, rice bran, liver, and cane molasses; the

residue contained growth-promoting but no anti-greying potency. Since the growth factor was also found in the active acid-ether extract, no conclusive evidence was furnished by this study for the separate existence of the anti-greying factor, but only for the possible existence of two separate growth factors.

Nielsen, Oleson & Elvehjem (326) described briefly a method by which the anti-greying vitamin may be crystallized from liver extract. Norite adsorbates were eluted and readsorbed, the final residue being extracted with acid ether, treated with barium hydroxide, and taken up in chloroform, from which solution crystals of high potency were obtained with ethyl acetate treatment. This crystalline product was active in daily doses of 15 μg. The chloroform extract was said to contain less than 1.5 μg. pantothenic acid in the daily dose of 70 μg. total solids which prevented greying in rats. Since factor W (317) was insoluble in chloroform, this was thought to separate it from the anti-greying factor. It should be pointed out, however, that prevention of greying in rats is not a wholly reliable assay method since some rats resist this symptom of the deficiency for long periods.

Morgan & Simms (327) described in detail the syndrome which was produced in rats by "filtrate factor" deficiency. These were chiefly greying of the fur in symmetrical patterns, skin lesions on shoulders and, in some cases, large skin ulcers which persisted for months. Filtrates from fuller's-earth–treated extracts of yeast, liver, cane molasses, and alfalfa cured these symptoms and restored growth in varying degrees, thus indicating the presence of two factors. Histological evidence of damage to the adrenal cortex, thyroids, and testes was also found in these deficient rats. Injection of adrenocortical extracts slowly produced darkening of the fur, but did not restore growth or cure the skin lesions. The earlier announcement (328) of these findings was the first suggestion of involvement of the adrenal glands in this deficiency disease and of relation of the syndrome to the phenomena of senescence. Guinea pigs, dogs, and silver foxes (329) were also found to exhibit the same symptoms.

Lunde & Kringstad (330) also described evidence for the existence of two filtrate factors required by the rat; both were soluble in phenol; one of the two, B_w, was insoluble in acid-ether and therefore was not pantothenic acid, but might be the same as factor W. The other, B_x, the anti-greying factor, appeared to be distinguished from B_w only by its slightly greater instability to heat.

Unna & Sampson (331) claimed clear-cut cure, as well as prevention, of the greying of fur in rats fed diets deficient in pantothenic acid by daily administration of at least 80 to 100 μg. of synthetic calcium pantothenate. They noted incomplete return of pigmentation, however, in many so-called cured cases, resulting in "pepper and salt" stippling of the black fur.

Williams (332), on the other hand, reported complete failure of both natural concentrates of barium pantothenate and pure calcium pantothenate in daily doses of 100 μg. for either prevention or cure of greying in rats. Care was taken in this experiment to supply all the possible other vitamin needs, such as choline, α-tocopherol, vitamin K, ascorbic acid, unsaturated fatty acids, and nicotinic acid. Rate of growth and length of life of the animal were reported as much enhanced by the pantothenate in spite of persistent greyness of fur. The lavish supply of essentials other than the anti-greying substance has been noted by others as favorable to greying. This is particularly true of the supply of pyridoxin and nicotinic acid (327).

Two reports appeared concerning the greying of fur in anemic rats. Free (333) concluded that greying of black rat hair may occur from a vitamin deficiency or from lack of the minerals iron, copper, and manganese, and that cure in either case can be effected only by provision of the missing dietary constituents. Since milk is poor both in filtrate factors and in these mineral elements, rats fed whole milk diets were especially susceptible to the condition. However, no evidence has as yet been advanced which connects the depigmentation directly with the nutritional anemia of milk-fed rats. Gorter (334) has maintained that copper is the specific mineral element which prevents depigmentation of the hair of rats and cats, not zinc, as had been claimed by Stirn, Elvehjem & Hart (335) for rats. In the light of recent events, it seems clear that the zinc-deficient and control rats of these investigators did not receive adequate amounts of several of the B vitamins. Gorter also suggested that the anemia and depigmentation produced by copper deficiency are not correlated and that the anemia results from a more rigorous deficiency than does the depigmentation.

There is at this time no conclusive evidence as to the nature of the mechanism which prevents or cures the greying of hair produced by filtrate factor deficiency. Pantothenic acid is clearly involved but is not adequate alone to maintain normal hair pigmentation. No clue as to the mechanism of pigment failure has been

unearthed, but the concurrent adrenal, testicular, liver, and kidney lesions point to a profound disturbance of tissue chemistry not unlike that seen in thiamin deficiency.

It is possible that too little attention has been paid to the interrelation or balance among these B vitamins, either in excesses or in deficiencies. The appearance of one set of deficiency symptoms after another in pellagra patients treated with single vitamins has been noted more than once, and rats which are pyridoxin deficient have been found resistant to greying (330, 336).

Pantothenic acid and lower organisms.—Stimulation of growth and acid production in *Lactobacillus casei* are the basis of methods for assay of pantothenic acid preparations (298, 312). Other microorganisms for the growth of which pantothenic acid was found essential were some members of the *Pasteurella* group (337), pneumococcus (338), *Proteus morganii* (339), *C. diphtheriae gravis,* some *intermedius* but not *mitis* strains (340), and three strains of yeast (228). Apparently certain organisms can synthesize β-alanine and others the acid fragment of pantothenic acid, a finding similar to that seen in the case of the two parts of the thiamin molecule.

Mosquito larvae appear to require for growth to the adult stage pantothenic acid, pyridoxin, flavin nucleotide and killed yeast (227).

Rat dermatitis, growth, and pantothenic acid.—Supplee *et al.* (208) and Richardson & Hogan (341) concluded that the dermatitis of rats deprived of both pyridoxin and pantothenic acid (or filtrate factors) could be cured only by administration of both missing factors. The skin conditions, in their opinion, were alike in both deficiencies. Chick, Macrae & Worden (200) found differences between the dermatitis produced by these two deficiencies. Unna (342) reported specific skin and fur disturbances in rats lacking filtrate factors; slow recovery from these symptoms with 50 μg. per day of pantothenic acid was noted. Prophylactic doses of 80 μg. seemed enough for optimal growth. Liver concentrates were superior to equal doses of pantothenic acid alone.

Pantothenic acid (and/or filtrate factors) in canine nutrition.—Fouts, Helmer & Lepkovsky (195) fed adult dogs a synthetic diet, supplemented with thiamin, riboflavin, pyridoxin, and nicotinic acid but deficient in the filtrate factors. The dogs developed gastrointestinal symptoms and died after 87 to 289 days. Some of the animals showed skin or gastrointestinal ulcerations and all had fatty

livers. Plasma chlorides and serum potassium were determined in three of the animals, and the conclusion was reached that there was no damage to the adrenal cortex, such as had been suggested by Morgan & Simms (328) ; but inspection of the data reveals a steady decrease in plasma chloride concentration with the progress of the deficiency. Apparently the adrenals were not examined post mortem. There was no involvement of the nervous system such as had been noted by Chick *et al.* (343) and Wintrobe *et al.* (344) in pigs and by Phillips & Engel in chicks (315). The use of adult animals may have complicated the deficiency syndrome.

McKibbin *et al.* (345) fed young mongrel dogs a purified diet supplemented with crystalline thiamin, riboflavin, and nicotinic acid and added various liver extract fractions in an attempt to discover their other B-vitamin needs. Anemia occurred due to pyridoxin deficiency as had been seen previously by Fouts *et al.* (346) and failure in growth occurred unless a filtrate factor which they thought might be "factor W" was also supplied. Pantothenic acid, they thought, was not required since they obtained good growth (for forty-three days) in two dogs fed the alcohol-ether precipitate fraction of liver extract, which was not protective against chick dermatitis. Growth only (which is not always reliable in mongrel dogs) was used as criterion of success, and in general only short periods were used, seven to ninety-five days.

McKibbin *et al.* (347) later tried a similar experiment but supplied pyridoxin as well as thiamin, riboflavin, and nicotinic acid along with the purified diet. They found that pantothenic acid and another alkali-labile factor, as well as factor W, were needed for growth of the young mongrel puppies. Again the test periods used were short, three to forty-three days. It seems clear that dogs require one or more B vitamins other than thiamin, pyridoxin, riboflavin, nicotinic acid, and pantothenic acid.

Borson & Mettier (196), in a study of the hypochromic microcytic anemia produced by pyridoxin deficiency in young dogs, found that pyridoxin did not completely relieve the anemia unless filtrate factors were also given. They were not convinced that the dog filtrate factor was identical with either the chick antidermatitis factor or the rat-growth filtrate factor. Morgan & Simms (327) also used purified diets and crystalline vitamin supplements with young dogs maintained for long periods and found them more susceptible to the filtrate factor deficiency when nicotinic acid was given than when it

was withheld. Greying of the hair occurred as also in young silver foxes (329) and guinea pigs. Large mottled thymuses were seen in some of these deficient animals, a phenomenon noted also by Mc-Kibbin *et al.* (345) in two of their dogs which were deficient in filtrate factor. Lunde & Kringstad (348) also found silver foxes peculiarly susceptible to the depigmentation resulting from this deficiency.

Adrenal necrosis.—The first mention of adrenal gland damage as due to filtrate factor deficiency was that of Morgan & Simms (328), who noted cortex atrophy, preceded in the earlier stages of the deficiency by excessive vascularity. This was followed by that of Daft & Sebrell (349) who, in a search for blood changes, found hemorrhagic necrosis in fourteen of seventeen rats fed diets deficient in filtrate factor and in some cases deficient also in pyridoxin. Nine rats given a rice bran filtrate preparation had no adrenal pathology. Nelson (350) reported varying amounts of adrenal damage in forty-four out of seventy-four rats used in a study in which various deficient but unidentified diets were fed. Daft *et al.* (351) studied histologically the adrenal glands of sixteen rats kept on a diet deficient in filtrate factor for eight to twelve weks and of twenty-eight rats treated similarly except that during the last two weeks they were given a total of 0.6 to 2.8 mg. synthetic pantothenic acid. Ten of the untreated rats had varying degrees and kinds of adrenal lesions and several also had abnormal testicular function; only one of the treated animals showed any lesions. The conclusion appeared manifest that pantothenic acid in an astonishingly short time specifically cured the adrenal lesions of "filtrate factor deficiency" in rats. Ashburn (214) studied the histopathology of some of these rats, noting the evidence of repair in adrenals, testicular function, and cartilage hypoplasia of the rats treated with pantothenic acid. The suggestion is made that these animals had suffered partial or complete adrenal insufficiency on the deficient diet. This curative action of pantothenic acid and the adrenal-damaging effect of the deficiency were confirmed in two other laboratories (352, 353).

Pantothenic acid in human nutrition.—The method of Pennington *et al.* (312) was applied by Stanbery, Snell & Spies (354) to the determination of pantothenic acid in human blood. They found that in eighteen normal persons the blood level fell within the limits of 0.19 to 0.32 μg. per cc., but in persons with various deficiency diseases such as pellagra, beriberi, or ariboflavinosis, the limits were

0.05 to 0.09 µg. per cc. The authors suggest that this may mean that pantothenic acid is important in human nutrition.

Vitamins and lactation.—With the advent of crystalline thiamin, riboflavin, and pyridoxin, a new attack on the difficult problem of the vitamin requirements during lactation became possible. Sure (355) found that rats on purified diets could rear young successfully if given daily 120 µg. thiamin, 120 µg. riboflavin, 50 µg. pyridoxin, 15 mg. choline chloride, 6 mg. nicotinic acid, and 1 cc. of a "factor W" solution equivalent to 1 gm. liver extract. There was complete failure without the "factor W." This preparation undoubtedly contained pantothenic acid and any other filtrate factors present in the liver extract. Morgan & Simms (328) noted that their "filtrate factor" was specifically required for lactation. Nakahara *et al.* (356) claimed that their vitamin-L deficiency (357) was independent of filtrate factor deficiency since their basal diet contained acid earth adsorbate of brewer's yeast and polished rice powder and was adequate for growth of young rats. Factor L_1 only was supplied by liver filtrate and L_2 by baker's yeast filtrate, and both were needed to secure normal lactation, but brewer's yeast filtrate (used by Morgan & Simms) contained both. However, Sure's (355) factor W must have contained only L_1. Nakahara *et al.* (358) have later reported that with dextrin instead of polished rice in the diet, lactation was successful with a supplement of liver filtrate only, thus indicating that L_2 must be produced by the "intestinal yeasts." However, even with both L_1 and L_2 only 50 to 60 per cent of the young were weaned.

Jukes (359) used crystalline vitamins only (per 100 gm. of diet: 2.8 mg. pantothenic acid, 1.0 mg. nicotinic acid, 0.5 mg. riboflavin, 0.2 mg. thiamin, 0.2 mg. pyridoxin, 50 mg. choline chloride). Growth, 60 to 80 per cent of normal, was obtained with this diet in the first generation as well as in the second, and about 50 per cent of the young survived, but greying of fur occurred in the males. Without the pantothenic acid there was no survival of young.

A crucial comparison of the importance of the various B vitamins for lactation in rats may soon become possible, but obviously everything needed is not yet identified.

Other B Factors

Biotin, Vitamin H, and Coenzyme R.—Kögl & Tönnis (360) in 1936 announced the isolation in crystalline form of a factor "biotin"

essential for yeast growth. Later, West & Wilson (361) and Nilsson *et al.* (362) concluded that biotin and coenzyme R were probably identical. This coenzyme had been shown by Allison *et al.* (363) to be an essential growth and respiration factor for many strains of the legume nodule organism *Rhizobium*. György *et al.* (364) noted the similarity between the properties of vitamin H (the anti-egg white injury factor) and those of biotin and coenzyme R. They found that purified vitamin-H preparations had parallel activity as both factors. Meanwhile Snell *et al.* (365) developed a highly sensitive bioassay for biotin and suggested, because of its rate of destruction by nitrous acid, that it may be an amino acid. Du Vigneaud *et al.* (321) discovered that Kögl's preparation of biotin methyl ester (m.p. 148°) had a vitamin-H activity of 10,000 units per mg. as contrasted with 215 units per mg. for their most potent vitamin-H concentrates. The group then (366) isolated biotin methyl ester (m.p. 166°–167°) from a liver concentrate with vitamin-H unitage of 27,000 per mg. and confirmed with this preparation the identity of biotin with coenzyme R. Eakin *et al.* (367, 368), suspecting from experiments with chicks that the injury caused by egg white might not be due to toxicity but indirectly to the action of the egg white in making the biotin of the diet unavailable, succeeded in obtaining inactivation of biotin by egg albumin *in vitro*. They effected a thousand-fold concentration of the egg white injury factor.

Thus was a new identification of coenzyme and vitamin activity accomplished.

The grass juice factor.—Randle, Sober & Kohler (376) have found animal tissues to be a poor source of the grass juice factor (required by guinea pigs) and report great variations in the amount in plant materials. Young, rapidly metabolizing plant tissues were said to be much richer in this factor than older and more mature material. They also reported that the factor is labile to oxidation and that glucuronic acid, narcotine, and calcium eriodictate fed to guinea pigs did not exert activity as grass juice factor.

Winegar & Pearson (377), in supplementing a typical pellagra-producing diet fed to rabbits with grass juice factor, were able to bring about some improvement, but normal performance did not result even when riboflavin and nicotinic acid were also fed.

Mouse factors.—Two additional factors required by mice have been reported. Woolley (378) found that mice fed a purified diet containing the known factors of the B complex ceased to grow and

became hairless over the trunk. He later (379) identified the missing substance as inositol, apparently present in liver in alkali-labile combination with a large molecule. Norris & Hauschildt (380) also described a similar deficiency disease in mice with loss of hair and dry scaly skin.

New bird factors.—A new deficiency disease in chicks, resulting in anemia, was reported by Hogan & Parrott (381) while a similar condition was found in pigeons (382). None of the recognized factors appeared to be effective in curing the anemia. A somewhat similar pigeon disease was described by Dameshek & Myerson (383).

The chick growth factor found in polished rice but apparently not in yeast (384, 385) was identified by Almquist *et al.* (386) as glycine and chondroitin. The glucuronic acid and not the galactosamine component of the chondroitin was recognized as the essential factor. Thus glycine, arginine, and glucuronic acid (or a similarly acting substance) are chick growth factors. Factor U, previously described as needed by chicks, was found to be partly pyridoxin and partly an unknown eluate factor (387). Schumacher *et al.* (388, 389) described two new growth factors, R and S, extractable by dilute acid from brewer's yeast and separated by precipitation of one with alcohol. One or the other of these may be the same as the residue of factor U, as may also be the factor of Bauernfeind & Norris (314).

Choline.—Recent events make it reasonable to include choline provisionally among the water-soluble vitamins. Up to 1940 choline had been shown to have (*a*) lipotropic activity, promoting phospholipid turnover and preventing fatty liver production, (*b*) protective action against hemorrhagic kidney degeneration, and (*c*) an important function in the conversion of homocystine to methionine. Jukes (390) has now shown that choline with manganese acts as a preventive of perosis in turkeys and chickens. Griffith & Wade (391) have confirmed and extended their study of the hemorrhagic kidney lesions and fatty livers produced in young rats by choline deficiency, particularly with a cystine-rich diet. Young animals and males were found to be more sensitive than older rats and females (392). These lesions are similar to those described by Curtis *et al.* (393) as due to cystine poisoning in rats and are mitigated by choline and methionine as well as by low-fat and low-cholesterol diets (394).

A clue to this action of choline may be seen in the interesting discovery by du Vigneaud *et al.* (395) that homocystine can replace methionine in the diet of the rat, only in the presence of choline or

betaine. This involves the transfer of the methyl group from choline to homocystine, and presumably of the reverse action. The latter was demonstrated by du Vigneaud *et al.* (396) by the production in the rat of deuterium-containing choline from deuterium-containing methionine. Methionine is thus seen to be an important methylating agent as presumably must also be true of choline.

Sure (397) has suggested that choline is needed for lactation and growth of rats. Daily doses of 15 mg. choline chloride were needed to maintain satisfactory lactation and growth of the young and prevention of sudden paralysis and kidney damage in the young. György & Goldblatt (398) also confirmed Griffith's (392) finding of the need for choline by young weaned rats. The antagonism between cystine and choline, observed in fatty liver studies, appears somewhat confused by their effects on lactation since Daggs (399) has found lactogogic activity in cystine and cysteine.

The quantity of choline required and its replaceability by methionine, betaine or other substances may argue against its inclusion among the vitamins. Its lipotropic activity has been explained by Perlman & Chaikoff (400) as due to its speeding-up effect upon liver phospholipid turnover, an effect now shown to be paralleled by methionine and, strangely enough, also by cystine and cysteine (401).

SYNTHESIS OF VITAMINS IN THE RUMEN

The apparent independence of cattle of the need for B vitamins which was reported by Bechdel *et al.* (369) some years ago has been generally assumed to be due to bacterial synthesis of these factors in the rumen. McElroy & Goss have now published quantitative studies of the production of several of the members of the B complex in the rumen of sheep and cows. Thiamin, pantothenic acid (370), riboflavin, vitamin K (371), biotin (372), and pyridoxin (373) were found to be present in the rumen contents of a fistulated cow and of sheep which had been fed experimental diets deficient in these factors.

Wegner *et al.* (374), likewise using a fistulated heifer, proved the synthesis in the rumen of thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, and biotin. Addition of pure thiamin to the deficient ration resulted in further increases in the bacterial

synthesis and no destruction of the added thiamin. Ascorbic acid, however, was found by Knight *et al.* (375) to be rapidly and completely destroyed in the rumen of the cow.

ASCORBIC ACID

A large volume of research, dealing with all phases of ascorbic acid chemistry and physiology, has appeared as usual. The most important practical phases of human utilization and need have been reviewed here but many valuable contributions could not be adequately covered. Fair agreement on methods for determination of status with reference to this vitamin has been reached, but little light has been thrown on its physiological role. There is no clear evidence that it enters into any of the respiratory enzyme systems nor that its function is chiefly detoxification. The value of excess or tissue saturation is attested chiefly by negative evidence. Most of the major problems remain unsolved in spite of the significant advances here listed.

The methods in use for determination of status and requirement.— The urinary excretion and plasma ascorbic acid values, particularly after administration of a single massive dose of the vitamin, are taken to indicate the status as to this factor. Other methods which have been proposed and variously criticized or supported are the intradermal reduction of dye and the capillary fragility or petechial test.

The excretion test.—Ludden & Wright (402) studied the effect of renal retention on vitamin-C saturation and excretion tests. A definite relation was established between the per cent of ascorbic acid excreted in the first 1½ hours, the first 5 hours, and the 24-hour output. A correction for renal retention errors in vitamin-C saturation tests may be made with the formula suggested.

Friedman *et al.* (403) also examined the clearance of ascorbic acid at low and normal plasma concentrations and concluded that at low levels clearance by the kidney is constant and independent of plasma concentration. Sherry *et al.* (404) examined the mechanism of excretion of vitamin C by the dog kidney by simultaneous creatinine and vitamin-C clearances. The latter appeared to be excreted by filtration and tubular reabsorption with limited maximal rate for reabsorption.

A curious relation between ovulation in women and minimum

excretion of ascorbic acid was suggested by Pillay (405) from determinations on eleven women. This might be a means of identifying the time of ovulation but it also introduces a complication into routine saturation tests.

The plasma ascorbic acid level.—Greenberg & Rinehart (406) and also Kassan & Roe (407) made a careful study of the stability of ascorbic acid in drawn blood. They confirmed the finding that the presence of red blood cells was protective but hemolysis was destructive, an effect not prevented by addition of cyanide. Heinemann & Hald (408) found that added ascorbic acid *in vitro* passed from serum to cells at 37° but not at 7° C., suggesting a metabolic reason rather than simple diffusion. Butler & Cushman (409) found that in subjects on a low vitamin intake the whole blood, and the white cells and platelets, might contain ascorbic acid or related substances even when the plasma concentration was zero. They concluded that the ascorbic acid content of white cells and platelets may be a better index of vitamin-C deficiency than the plasma values whereas ascorbic acid concentration in red cells or whole blood may be the best index of saturation.

Mindlin (410) measured the ascorbic acid of the plasma of newborn infants and their mothers and found the averages to be 1.16 and 0.42 mg. per cent respectively. Rapid rise and fall of the plasma level occurred in the infants following changes in intake but corresponding rapid depletion of tissues was hardly likely to have occurred. The author concluded that in newborn infants at least the plasma level is not a reliable index of vitamin-C status. The plasma concentration method was also used by Kajdi *et al.* (411) with intramuscular injection of 200 mg. ascorbic acid in children to assess the vitamin-C status. The relation of the postinjection increase to the initial concentration is offered as a satisfactory criterion.

Capillary test.—The capillary fragility test has not been widely used except in Sweden where it continues to give satisfaction. Ahlborg & Brante (412) found the petechial index and plasma ascorbic acid of children highly correlated whereas Rapaport *et al.* (413) declared that in 150 children they could detect correlation in only half of the number. Difs (414) concluded that neither capillary fragility, urinary saturation, nor plasma concentration yielded clear-cut evidence of vitamin-C status.

Intradermal test.—The intradermal test of Rotter was described as satisfactory (415) and is yielding results not correlated with plasma

ascorbic acid levels (416). Beck & Krieger (417) injected a solution of 0.0025 M dichlorophenolindophenol intracutaneously and noted that it was decolorized in ten minutes in normal subjects but in vitamin-C-deficient persons in thirteen minutes or more.

The human requirement.—The assessment of the optimum human requirement is still under discussion. The minimum amount needed to prevent actual scurvy has been estimated often enough, but the question of the criteria by which the most advantageous intake may be judged remains unanswered. Usually the amount is set at that needed to keep the plasma ascorbic acid at 1.0 mg. per cent or above, and the quantity which produces a sharply increased and sustained rate of excretion is accepted as allowing saturation of the tissues. So far no proof of definite advantage from such saturation has been forthcoming. Certainly no storage of the vitamin, such as may be obtained with vitamin A, results, for a sharp deficit occurs promptly on diets deficient in vitamin C regardless of previous saturation. This was shown by Lemmel (418) in a study of 110 children given 100 mg. ascorbic acid daily for six months. After four months, saturation as judged by excretion appeared to be complete. After fourteen days without the vitamin the vitamin deficit produced was as great in those saturated for months as in those saturated for only a few days.

Todhunter & Robbins (419), estimating from urine analysis the minimum intake of ascorbic acid required to maintain tissues in a state of complete saturation, found the daily requirement for three female adults to be from 1.6 to 1.7 mg. per kg. per day. The excretion was found to vary considerably for the same individual even with carefully controlled intake. Values above 1 mg. per cent ascorbic acid for blood plasma were obtained when the total daily intake was 60 mg. and more than 120 mg. daily were required to raise the blood plasma to 1.4 mg. per cent.

Ralli *et al.* (420), using three male adults, could not maintain the plasma level at 1.0 mg. per cent on less than 100 mg. daily; with 50 mg. doses plasma levels of 0.4 mg. per cent were obtained. They concluded that 100 mg. daily is the optimum for adults. Chen *et al.* (421) studied four adult male Chinese patients with scurvy and found their requirement to range from 1.46 to 1.68 mg. per kg. per day for maintenance of the plasma level at 1.0 mg. per cent and excretion rate above the basal level. This is in good agreement with Todhunter & Robbins (419).

Effects of deficiency.—Crandon & Lund (422, 423) placed a subject on a diet deficient in ascorbic acid for six months. The plasma ascorbic acid fell to a low level within 10 days and to zero in 30 days. Thirteen weeks from this time the first clinical evidence of scurvy appeared. The earliest clinical manifestations of scurvy were hyperkeratotic papules, noted about 132 days after the diet was instituted. The perifollicular hemorrhages of scurvy appeared a month later. The ascorbic acid value of the white cell platelet layer of the centrifuged blood fell to zero only at this time. As suggested by Butler & Cushman (409) this value appeared to be better correlated with clinical scurvy than the plasma value. There was loss of weight and lowered blood pressure but no anemia. Failure of wound healing was not noted until 141 days after the experiment started. Disappearance of all symptoms of scurvy was rapid following intravenous injection of ascorbic acid, with return of the plasma level to the normal 1 mg. per cent in 4 days and with only 4 gm. injected. This study is particularly interesting because it gives an accurate picture of a human deficiency disease under controlled conditions. The authors suggest that low plasma ascorbic acid levels indicate only the degree of saturation and are a poor index of deficiency.

A similar heroic study was made by Rietschel & Mensching (424) with quite similar results. The subject took a vitamin-C-free diet for 160 days with no symptoms for 120 days and no loss of weight.

Bartlett *et al.* (425) found that the fasting level of plasma ascorbic acid after an operation showed a consistent drop with a gradual return to the preoperative value. There was no increase in the urinary ascorbic acid after the operation, even following the intravenous administration of 1 gm. of ascorbic acid. More rapid clearance from fasting blood of ascorbic acid administered intravenously after the operation was possibly dependent upon an increased need for this substance in the process of tissue repair and wound healing. Certainly, slowing of wound healing appears to be one of the concomitants of vitamin-C deficiency.

Abt *et al.* (426) concluded from a study of ascorbic acid in the stools, plasma, and urine of infants that large amounts of orally administered ascorbic acid may be excreted in the stools following catharsis and during acute diarrhea. The increased fecal excretion of orally administered ascorbic acid during acute diarrhea in infants points to its failure of absorption in the intestinal tract and explains the low blood plasma values and low urinary excretion.

Ascorbic acid therapy.—The effects of ascorbic acid administration have again been studied in rheumatoid conditions (427), in tuberculosis (428, 429, 430, 431), in peptic ulcer (432), in fever (433, 434), in agranulocytosis (435), and in epilepsy (436). In nearly all of these conditions the ascorbic acid of the plasma was found to be low and some clinical advantage resulted from administration of enough of the substance to raise it to a normal level. However, no curative effect on the pathology was reported.

Jenovese *et al.* (437), in six patients with Addison's disease, found that the plasma ascorbic acid level was normal yet the urinary excretion low. This was taken to mean that a low rate of excretion does not necessarily indicate an ascorbic acid deficiency in this disease.

Ascorbic acid in foods.—Studies of ascorbic acid in various foods, cooked and raw, have appeared. The rich content of berries was again confirmed (438). Other reports deal with effects of maturation and storage on the ascorbic acid content of citrus fruits (439); of diet and injection of ascorbic acid on its concentration in goats' milk (440); a better method for determination of ascorbic acid in evaporated and powdered milks (441); effect of the age of the cow and stage of lactation on ascorbic acid of milk (442); of varietal differences in strawberries (443), cabbage (444), and parsnips (445); and the effect of cooking on turnip greens (446), and on quick-frozen vegetables (447).

On human subjects Clayton & Folsom (448) used potatoes as a source of ascorbic acid, for comparison with synthetic ascorbic acid, as did Todhunter & Fatzer (449) with red raspberries. In both cases the blood plasma level and rate of excretion indicated equally good utilization of the natural and synthetic sources.

Studies of the mechanism of ascorbic acid autoxidation have appeared with copper and iron implicated as chief catalysts (450, 451). The loss of ascorbic acid and damage to the flavor of milk resulting from oxidation catalyzed by copper or light exposure have received much attention. Opaque containers to protect the milk from light have been suggested (452) and considered unnecessary (453). Glutathione, 25 mg. per cent, added to previously heated milk was found by Gould (454) to inhibit copper-induced oxidation of ascorbic acid. Pure ascorbic acid added to milk was also found by Swanson & Sommer (455) to inhibit production of oxidized flavor. Woessner *et al.* (453) investigated the fate of ascorbic acid added to milk

under commercial conditions. Rigid exclusion of copper and light were found necessary for retention of ascorbic acid in fortified milk. According to Hand *et al.* (456) the simple removal of dissolved oxygen by vacuum cooling prevents loss of ascorbic acid in both raw and pasteurized milk even when the milk is exposed to copper and light.

Ascorbic acid and tyrosine metabolism.—It was discovered last year that alcaptonuria can be produced in guinea pigs on low ascorbic acid intake by feeding tyrosine (457). Apparently the metabolism of tyrosine and dihydroxyphenylalanine increased ascorbic acid requirements. Levine *et al.* (458) found that premature infants fed cow's milk excreted hydroxy and ketophenyl compounds, but that such excretion ceased on administration of 50 to 200 mg. ascorbic acid. The response appeared to be conditioned by tissue saturation. Continuing this line of research Sealock & Silberstein (459) fed 0.5 gm. or more of tyrosine daily to guinea pigs on a diet deficient in ascorbic acid, and observed excretion of homogentisic, *p*-hydroxyphenylpyruvic and *p*-hydroxyphenylacetic acids. The administration of 10 mg. or less of *l*-ascorbic acid daily to these animals completely prevented the excretion of these metabolites. It was found however (460) that the ingestion of 1 to 4 gm. ascorbic acid daily had no effect on the excretion of homogentisic acid in the case of a patient excreting an average of 7 gm. of this metabolite per day. The mechanism of hereditary alcaptonuria is apparently not the same as that induced in ascorbic acid–deficient guinea pigs.

Rothman (461), in the course of an investigation into the formation of melanin from tyrosine by ultraviolet irradiation, found that ascorbic acid furthered the actinic transformation of tyrosine or any *p*-monohydroxyphenyl derivative into dihydroxyphenylalanine or the corresponding catechol derivative but inhibited any further oxidation. The stabilization of dihydroxyphenylalanine by ascorbic acid and prevention of further oxidation by irradiation were absolute if an excess of ascorbic acid were present. These findings are thought to indicate that any kind of physiologic or pathologic pigmentation can be prevented by ascorbic acid if its concentration in the tissue is great enough. The presence of ascorbic acid in the suprarenal medulla may therefore have a double importance, formation of catechol precursors of epinephrine from phenolic constituents of protein and stabilization of epinephrine after it is formed.

Metabolism of the vitamin.—Ball (462) found that 2-keto-*l*-gulonic

acid when fed to guinea pigs possessed practically no antiscorbutic property. Thus no regeneration of 2,3-diketo-*l*-gulonic acid, an oxidation product of ascorbic acid, appears likely in the animal body. This suggests that the opening of the lactone ring in ascorbic acid is tantamount to loss of antiscorbutic properties. The simultaneous loss of reducing and antiscorbutic properties on opening of the lactone ring further suggests that the reducing action of ascorbic acid is in some way associated with its biological function.

Since the iminazole ring of thioneine had been shown (463) to be split by ascorbic acid *in vitro*, Greenblatt & Pecker (464) studied this action *in vivo* in rabbits, guinea pigs, and man. They found that blood thioneine was not affected in any case nor was blood uric acid in man. Since histamine retained its stimulating effect on the gastric secretion it was assumed also to be untouched.

Some studies relating the role of ascorbic acid in metabolism to the glycogen–lactic acid cycle have appeared. Milhorat *et al.* (465), using dogs, explained the increased creatinuria and ascorbic acid output caused by shivering and epinephrine treatment as due to failure of phosphocreatine resynthesis. The liver was also considered to be involved in ascorbic acid metabolism, probably through its glycogenetic function (466). The findings of Ralli & Sherry (467) on the depression by insulin of the fasting plasma level and the rate of excretion of ascorbic acid in normal and diabetic dogs point to a similar connection.

Thaddea & Scharsach (468) presented further evidence of a possible antagonism between ascorbic acid and thyroid function, and Leblond & Chamorro (469) demonstrated that hypophysectomized rats can still synthesize the vitamin. Holtz (470) continued his studies of the protein-bound form of the vitamin in both plants and animals, distinguished from the free form by its resistance to irreversible oxidations. The amount of the bound form seemed to remain unchanged in ascorbic acid–deficient animals and may represent storage.

Ascorbic acid and detoxification.—Previous experimental work by Longenecker and associates has shown that ascorbic acid excretion by rats may be increased by feeding a variety of compounds, such as terpene-like cyclic ketones, alcohols, a lipid obtained from alfalfa meal, and certain aliphatic ketones and alcohols. Longenecker, Fricke & King (471) found that many compounds used as nerve depressants greatly accelerate ascorbic acid excretion. No evidence was obtained to indicate that the urinary ascorbic acid was conjugated with any

of the toxic substances fed but its endogenous production appeared to be related to the animals' detoxification processes.

Samuels *et al.* (472) found that salicylates fed to rats and guinea pigs rapidly caused an increased excretion of ascorbic acid by both species; this was not due to diuresis or to renal damage.

Torrance (473) saw no significant difference between the ascorbic acid content of the suprarenals of guinea pigs injected with a sublethal dose of diphtheria toxin and that of control animals. The smallest dose which affected the ascorbic acid content of the suprarenals was the minimum lethal dose. No evidence of direct combination of ascorbic acid and diphtheria toxin was found. King *et al.* (474) noted a protective effect against the tooth injury caused by diphtheria toxin in guinea pigs when the ascorbic acid intake was raised to three times the amount needed for normal growth and tooth structure. However, this was not taken as evidence for a direct detoxifying effect by the vitamin. A further study of the condition and rate of deposit of dentine in the incisors of normal guinea pigs by Boyle *et al.* (475) indicated close correlation with the intake of ascorbic acid. Pillemer *et al.* (476) found that guinea pigs were protected against the neuroplumbism of lead poisoning by saturation rather than subclinical scurvy levels of ascorbic acid intake but detected no effect of the lead on ascorbic acid metabolism.

Citrin

Experimental studies in 1940 lent little support to the claim that citrin (hesperidine and eriodictyol glucoside) exerts an effect upon vascular permeability either with or without ascorbic acid. Guinea pigs were thought to show fewer hemorrhages when protected from scurvy by borderline doses of lemon juice than when given equivalent amounts of crystalline ascorbic acid (477). However Detrick *et al.* (478) and McHenry & Perry (479) saw no advantages to scorbutic guinea pigs when citrin was administered. Neuweiler (480) could find no citrin in human or cow's milk and none in the milk of women who received 50 mg. injections of this substance daily for six days. He thought this indicated that citrin has no physiological role.

On the other hand several clinical reports (481, 482, 483) indicate favorable effects from the use of citrin in purpura of allergic

and nutritional origin. Mechanical purpura was not affected. Scarborough (484), from a study of six cases of scurvy, concluded that two forms of subcutaneous bleeding of nutritional origin exist in man, one of which is due to ascorbic acid deficiency and the other to citrin deficiency. Hemorrhages in the latter were petechial in character, were accompanied by low capillary resistance, and were readily cured by citrus concentrates. None of the true scorbutic symptoms were affected by these preparations. The citrin problem remains unsolved.

LITERATURE CITED

THIAMIN

1. De Caro, L., *Boll. soc. ital. biol. sper.*, **15**, 203 (1940)
2. Jukes, T. H., and Heitman, H., *J. Nutrition*, **19**, 21 (1940)
3. Emerique-Blum, L., and Lwoff, A., *Bull. soc. chim. biol.*, **22**, 179 (1940)
4. Sinclair, H. M., *Biochem. J.*, **33**, 2027 (1939)
5. De Caro, L., and Butturini, L., *Boll. soc. ital. biol. sper.*, **15**, 207, 404 (1940)
6. Henry, K. M., Houston, J., Kon, S. K., and White, P., *J. Dairy Research*, **11**, 145 (1940)
7. Houston, J., Kon, S. K., and Thompson, S. Y., *J. Dairy Research*, **11**, 145 (1940)
8. Widenbauer, F., *Klin. Wochschr.*, **18**, 1613 (1939)
9. Booth, R. G., *J. Soc. Chem. Ind.*, **59**, 181 (1940)
10. Borson, H. J., *Ann. Internal Med.*, **14**, 1 (1940)
11. Ferrebee, J. W., and Carden, G. A., *J. Lab. Clin. Med.*, **25**, 1320 (1940)
12. Westenbrink, H. G. K., and Goudsmit, J., *Arch. néerland. physiol.*, **24**, 305 (1940)
13. Schultz, A. S., Atkin, L., and Frey, C. N., *J. Biol. Chem.*, **136**, 713 (1940)
14. Emmett, A. D., Peacock, G., and Brown, R. A., *J. Biol. Chem.*, **135**, 131 (1940)
15. Sakurai, Y., Inagaki, T., and Omori, S., *J. Agr. Chem. Soc. Japan*, **16**, 331 (1940)
16. Yang, E. F., and Platt, B. S., *Chinese J. Physiol.*, **14**, 259 (1939)
17. Ochoa, S., and Peters, R. A., *Biochem. J.*, **32**, 1501 (1938)
18. Lipschitz, M. A., Potter, V. R., and Elvehjem, C. A., *Biochem. J.*, **32**, 474 (1938)
19. Weil-Malherbe, H., *Biochem. J.*, **33**, 1997 (1939)
20. Westenbrink, H. G. K., and van Dorp, D. A., *Nature*, **145**, 465 (1940)

21. LIPTON, M. A., AND ELVEHJEM, C. A., *J. Biol. Chem.*, **136**, 637 (1940)
22. GOODHART, R. S., AND SINCLAIR, H. M., *Biochem. J.*, **33**, 1099 (1939)
23. GOODHART, R., *J. Biol. Chem.*, **135**, 77 (1940)
24. WESTENBRINK, H. G. K., *Enzymologia*, **8**, 97 (1940)
25. WESTENBRINK, H. G. K., *Compt. rend. trav. lab. Carlsberg*, **23**, 195 (1940)
26. WIDENBAUER, F., *Klin. Wochschr.*, **18**, 1392 (1939)
27. RITSERT, K., *Klin. Wochschr.*, **18**, 1370 (1939)
28. BANGA, I., OCHOA, S., AND PETERS, R. A., *Biochem. J.*, **33**, 1980 (1939)
29. KREBS, H. A., AND EGGLESTON, L. V., *Biochem. J.*, **34**, 442 (1940)
30. SOBER, H. A., LIPTON, M. A., AND ELVEHJEM, C. A., *J. Biol. Chem.*, **134**, 605 (1940)
31. OCHOA, S., *Nature*, **146**, 267 (1940)
32. STERN, K. G., AND MELNICK, J. L., *J. Biol. Chem.*, **131**, 597 (1939)
33. LIPMANN, F., *Nature*, **138**, 1097 (1936)
34. STERN, K. G., AND MELNICK, J. L., *J. Biol. Chem.*, **135**, 365 (1940)
35. BARRON, E. S. G., AND LYMAN, C. M., *Science*, **92**, 337 (1940)
36. PETERS, R. A., *Nature*, **146**, 387 (1940)
37. LUNDE, G., KRINGSTAD, H., AND OLSEN, A., *Angew. Chem.*, **53**, 123 (1940)
38. AUGHEY, E., AND DANIEL, E. P., *J. Nutrition*, **19**, 285 (1940)
39. ARNOLD, A., AND ELVEHJEM, C. A., *Food Research*, **4**, 547 (1939)
40. LEONG, P. C., *J. Malaya Branch Brit. Med. Assoc.*, **4**, 66 (1940)
41. PYKE, M., *Biochem. J.*, **34**, 330 (1940)
42. BORSOOK, H., BUCHANAN, E. R., HATCHER, J. B., YOST, D. M., AND McMILLAN, E., *Proc. Natl. Acad. Sci. U.S.*, **26**, 412 (1940)
43. LONGENECKER, H. E., GAVIN, G., AND McHENRY, E. W., *J. Biol. Chem.*, **134**, 693 (1940)
44. OLDHAM, H., AND SCHLUTZ, F. W., *J. Nutrition*, **19**, 569 (1940)
45. McHENRY, E. W., *Science*, **86**, 200 (1937)
46. KARRER, P., *Helv. Chim. Acta*, **20**, 369 (1937)
47. KASAHARA, M., KAKUSUI, T., AND NAN, S. S., *Klin. Wochschr.*, **17**, 1259 (1938)
48. SÄKER, G., *Klin. Wochschr.*, **19**, 99 (1940)
49. ROBIE, R., *Am. J. Surg.*, **48**, 398 (1940)
50. EULER, H. V., AND HÖGBERG, B., *Naturwissenschaften*, **28**, 29 (1940)
51. AWE, W., *Naturwissenschaften*, **28**, 92 (1940)
52. AHLSTRÖM, L., SCHLENK, F., AND EULER, H. V., *Naturwissenschaften*, **28**, 188 (1940)
53. BARNES, R. H., AND MacKAY, E. M., *Proc. Soc. Exptl. Biol. Med.*, **45**, 759 (1940)
54. ERSPAMER, V., *Boll. soc. ital. biol. sper.*, **14**, 655 (1939)
55. ERSPAMER, V., *Arch. intern. pharmacodynamie*, **63**, 385; **64**, 1 (1940)
56. DE SOLDATI, L., *Compt. rend. soc. biol.*, **133**, 739 (1940)
57. BIRKHÄUSER, H., AND SÜLLMAN, H., *Schweiz. med. Wochschr.*, **70**, 34 (1940)
58. MANN, P. J. G., AND QUASTEL, J. H., *Nature*, **145**, 856 (1940)
59. BERNHEIM, J., *J. Lab. Clin. Med.*, **26**, 20 (1940)
60. DIMICK, M. K., *J. Nutrition*, **19**, 605 (1940)

61. SWANK, R. L., *J. Exptl. Med.*, **71**, 683 (1940)
62. ALEXANDER, L., *Am. J. Path.*, **16**, 61 (1940)
63. DE CARO, L., AND BUTTURINI, L., *Boll. soc. ital. biol. sper.*, **15**, 406 (1940)
64. NITZESCU, I. I., AND IOANID, V., *Compt. rend. soc. biol.*, **133**, 490 (1940)
65. HERMANN, V. S., *Z. physiol. Chem.*, **262**, 95 (1939)
66. FORNAROLI, P., AND BONI, I., *Boll. soc. ital. biol. sper.*, **15**, 515 (1940)
67. FORNAROLI, P., AND BONI, I., *Boll. soc. ital. biol. sper.*, **15**, 218 (1940)
68. SHILS, M., DAY, H. G., AND McCOLLUM, E. V., *Science*, **91**, 341 (1940)
69. BANERJI, G. G., *Biochem. J.*, **34**, 1329 (1940)
70. ROBINSON, W. D., MELNICK, D., AND FIELD, JR., H., *J. Clin. Investigation*, **19**, 483 (1940)
71. DE SOLDATI, L., *Compt. rend. soc. biol.*, **133**, 323 (1940)
72. DE SOLDATI, L., *Compt. rend. soc. biol.*, **133**, 726 (1940)
73. HAYNES, F. W., AND WEISS, S., *Am. Heart J.*, **20**, 34 (1940)
74. MacDONALD, D. G. H., AND McHENRY, E. W., *Am. J. Physiol.*, **128**, 608 (1940)
75. AYKROYD, W. R., KRISHNAN, B. G., PASSMORE, R., AND SUNDARAROJAN, A. R., *Indian Med. Research Mem.*, No. 32 (1940)
76. VEDDER, E. B., *Am. J. Trop. Med.*, **20**, 625 (1940)
77. SCRIBA, K., *Beitr. path. Anat.*, **104**, 76 (1940)
78. EGGLETON, W. G. E., *Chinese J. Physiol.*, **15**, 33 (1940)
79. WEISS, S., *J. Am. Med. Assoc.*, **115**, 832 (1940)
80. PANNEKOEK-WESTENBURG, S. J. E., AND VAN VEEN, A. G., *Geneeskund. Tijdschr. Nederland-Indië*, **80**, 1774 (1940)
81. MAGYAR, I., *Z. Vitaminforsch.*, **10**, 32 (1940)
82. GOODHART, R., AND SINCLAIR, H. M., *J. Biol. Chem.*, **132**, 11 (1940)
83. MOLNÁR, S., AND HORÁNYI, M., *Klin. Wochschr.*, **19**, 204 (1940)
84. HILLS, G. M., *Biochem. J.*, **39**, 1966 (1939)
85. ROBINSON, W. D., MELNICK, D., AND FIELD, JR., H., *J. Clin. Investigation*, **19**, 399 (1940)
86. CARDEN, G. A., PROVINCE, W. D., AND FERREBEE, J. W., *Proc. Soc. Exptl. Biol. Med.*, **45**, 1 (1940)
87. MAGYAR, I., *Orvosi Hetilap*, **83**, 1071 (1939)
88. BORSON, H. J., *Ann. Internal Med.*, **14**, 1 (1940)
89. POLLACK, H., DOLGER, H., ELLENBERG, M., AND COHEN, S., *Proc. Soc. Exptl. Biol. Med.*, **44**, 98 (1940)
90. NAJAR, V. A., AND HOLT, JR., L. E., *Bull. Johns Hopkins Hosp.*, **67**, 107 (1940)
91. WANG, Y. L., AND YUDKIN, J., *Biochem. J.*, **34**, 343 (1940)
92. LEAGUE OF NATIONS COMMISSION, *Quart. Bull. Health Organization League Nations*, **7**, 474 (1938)
93. GUERRANT, N. B., AND DUTCHER, R. A., *J. Nutrition*, **20**, 589 (1940)
94. COWGILL, G. R., *J. Am. Med. Assoc.*, **113**, 2146 (1939)
95. SCHEUNERT, A., *Deut. med. Wochschr.*, **66**, 365 (1940)
96. BAKER, A. Z., AND WRIGHT, M. D., *Brit. Med. J.*, **1**, 599 (1936)
97. EDITORIAL, *J. Am. Med. Assoc.*, **115**, 1198 (1940)
98. KNOTT, E. M., *Proc. Soc. Exptl. Biol. Med.*, **45**, 765 (1940)

99. JOLLIFFE, N., *Intern. Clinics,* **4**, 46 (1938)
100. STIEBELING, H. K., AND PHIPARD, E. F., *U.S. Dept. Agr. Dept. Circ.,* No. 507 (1939)
101. STIEBELING, H. K., *J. Nutrition,* Suppl., **19**, 21 (1940)
102. HARRIS, L. J., *Lancet,* **II**, 539 (1940)
103. RUBENSTONE, A. I., MERANZE, D., AND MERANZE, T., *Penna. Med. J.,* **43**, 930 (1940)
104. BROWN, P. W., *Proc. Staff Meetings Mayo Clinic,* **15**, 215 (1940)
105. AYKROYD, W. R., *Nature,* **145**, 522 (1940)
106. SCHULTZ, A. S., ATKIN, L., AND FREY, C. N., *Cereal Chem.,* **16**, 1643 (1939)
107. TISDALL, F. F., *J. Am. Med. Assoc.,* **105**, 1580 (1935)
108. FOREIGN LETTER, *J. Am. Med. Assoc.,* **115**, 1903 (1940)
109. GRAHAM-LITTLE, E., *Lancet,* **II**, 176 (1940)
110. MORAN, T., AND DRUMMOND, J. C., *Nature,* **146**, 117 (1940)
111. BAILEY, C. H., *Northwestern Miller,* **202**, 23 (1940)
112. CHICK, H., *Lancet,* **II**, 511 (1940)
113. WILLIAMS, R. D., MASON, H. L., WILDER, R. M., AND SMITH, F. B., *Arch. Internal Med.,* **66**, 785 (1940)
114. FANTUS, B., *J. Am. Med. Assoc.,* **115**, 450 (1940)
115. KIENE, H. E., STREITWIESER, R. J., AND MILLER, H., *J. Am. Med. Assoc.,* **114**, 2191 (1940)
116. EDITORIAL, *J. Am. Med. Assoc.,* **115**, 1890 (1940)
117. JOLLIFFE, N., *Quart. J. Studies Alcohol,* **1**, 74 (1940)
118. JELLINEK, E. M., AND JOLLIFFE, N., *Quart. J. Studies Alcohol,* **1**, 110 (1940)
119. SIDDALL, A. C., *Am. J. Obstet. Gynecol.,* **39**, 818 (1940)
120. WILLIAMS, P. F., *Am. J. Surg.,* **40**, 118 (1940)
121. BEAN, W. B., AND SPIES, T. D., *J. Am. Med. Assoc.,* **115**, 1078 (1940)
122. ARING, C. D., EVANS, J. P., AND SPIES, T. D., *J. Am. Med. Assoc.,* **113**, 2105 (1939)
123. VORHAUS, M. J., *Am. J. Med. Sci.,* **198**, 837 (1939)
124. MEIKLEJOHN, A. P., *New Engl. J. Med.,* **223**, 265 (1940)
125. SPIES, T. D., HIGHTOWER, D. P., AND HUBBARD, L. H., *J. Am. Med. Assoc.,* **115**, 292 (1940)
126. FROSTIG, J. P., AND SPIES, T. D., *Am. J. Med. Sci.,* **199**, 268 (1940)
127. LEWY, F. H., SPIES, T. D., AND ARING, C. D., *Am. J. Med. Sci.,* **199**, 840 (1940)
128. FEIN, H. D., ROLLI, E. P., AND JOLLIFFE, N., *J. Am. Med. Assoc.,* **115**, 1973 (1940)
129. SALVESON, O., *Nord. Med.,* **5**, 279 (1940)
130. BRAENDSTRUP, P., *Ugeskrift Laeger,* **102**, 95 (1940)
131. SELFRIDGE, G., *Calif. Western Med.,* **52**, 214 (1940)
132. BRANDENBERG, K. C., *Arch. Otolaryngol.,* **31**, 189 (1940)
133. CHILDREY, J. H., *Laryngoscope,* **50**, 648 (1940)
134. OCHSNER, A., AND SMITH, M. C., *J. Am. Med. Assoc.,* **114**, 947 (1940)
135. GORDON, A., *Med. Record,* **151**, 273 (1940)

136. IMLER, A. E., AND WAMMOCK, H., *Am. J. Roentgenol. Radium Therapy,* **43,** 243 (1940)
137. MOORE, M. J., *Arch. Internal Med.,* **65,** 1 (1940)
138. BORSOOK, H., KREMERS, M. Y., AND WIGGINS, C. G., *J. Am. Med. Assoc.,* **114,** 1942 (1940)
139. WILLIAMS, P., SHAPIRO, B. G., AND BARTELOT, R., *Lancet,* **I,** 76 (1940)
140. GRETTON-WATSON, B. G., *Lancet,* **I,** 244 (1940)
141. JANKELSON, I. R., *Am. J. Digestive Diseases,* **7,** 252 (1940)
142. BERNSTEIN, A., *Calif. Western Med.,* **52,** 69 (1940)
143. DIECKHOFF, J., *Monatschr. Kinderheilk.,* **82,** 53 (1940)
144. RUBIN, M. A., *Proc. Soc. Exptl. Biol. Med.,* **42,** 440 (1940)
145. McCORMICK, W. J., *Med. Record,* **151,** 282 (1940)
146. LÖWENSTEIN, W., AND NEUMANN, H., *Klin. Wochschr.,* **19,** 327 (1940)
147. SMITH, K. A., AND MASON, H. L., *Proc. Staff Meetings Mayo Clinic,* **15,** 529 (1940)

RIBOFLAVIN

148. SCHUMACHER, A. E., AND HEUSER, G. F., *Ind. Eng. Chem., Anal. Ed.,* **12,** 203 (1940)
149. KEMMERER, A. R., *J. Assoc. Official Agr. Chem.,* **23,** 346 (1940)
150. FERREBEE, J., *J. Clin. Investigation,* **19,** 251 (1940)
151. HODSON, A. Z., AND NORRIS, L. C., *J. Biol. Chem.,* **131,** 621 (1939)
152. FEENEY, R. E., AND STRONG, F. M., *J. Biol. Chem.,* **133,** 31 (1940)
153. FRASER, H. F., TOPPING, N. H., AND ISBELL, H., *U.S. Pub. Health Repts.,* **55,** 280 (1940)
154. SNELL, E. E., AND STRONG, F. M., *Ind. Eng. Chem., Anal. Ed.,* **11,** 346 (1939)
155. EL SADR, M. M., MACRAE, T. F., AND WORK, C. E., *Biochem. J.,* **34,** 601 (1940)
156. WAGNER, J. R., AXELROD, A. E., LIPTON, M. A., AND ELVEHJEM, C. A., *J. Biol. Chem.,* **136,** 357 (1940)
157. CLARKE, M. F., LECHYKA, M., AND COOK, C. A., *J. Nutrition,* **20,** 133 (1940)
158. HENRY, K. M., HOUSTON, J., AND KON, S. K., *Biochem. J.,* **34,** 607 (1940)
159. FIXSEN, M. A. B., AND ROSCOE, M. H., *Nutrition Abstracts & Revs.,* **9,** 795 (1940)
160. KUNERTH, B. L., AND RIDDELL, W. H., *Trans. Kansas Acad. Sci.,* **42,** 231 (1939)
161. SUMI, M., AND AKO, T., *Bull. Inst. Phys. Chem. Research (Tokyo),* **19,** 240 (1940)
162. SAFFRY, O. B., COX, H. S., KUNERTH, B. L., AND KRAMER, M. M., *J. Nutrition,* **20,** 169 (1940)
163. SHAW, G. E., *Quart. J. Pharm. Pharmacol.,* **12,** 541 (1939)
164. HODSON, A. Z., *Food Research,* **5,** 395 (1940)
165. POE, C. F., AND HARTMAN, L., *Univ. Colo. Studies, Ser. D,* **1,** 43 (1940)

166. HUNT, C. H., AND BETHKE, P. M., *J. Nutrition, 20, 175 (1940)

167. HODSON, A. Z., *J. Nutrition, 20, 377 (1940)

168. YAMASAKI, I., *Proc. Imp. Acad. (Tokyo), 16, 6 (1940)

169. MINIBECK, H., AND VERZAR, F., *Z. Vitaminforsch., 10, 79 (1940)

170. YUDKIN, A. M., AND GEER, H. A., *Arch. Ophthalmol. (Chicago), 23, 28 (1940)

171. AXELROD, A. E., LIPTON, M. A., AND ELVEHJEM, C. A., *Am. J. Physiol., 128, 703 (1940)

172. HUGHES, E. H., *J. Nutrition, 20, 233 (1940)

173. ENGEL, R. W., PHILLIPS, P. H., AND HALPIN, J. G., *Poultry Sci., 19, 135 (1940)

174. KAHLER, H., AND DAVIS, E. F., *Proc. Soc. Exptl. Biol. Med., 44, 604 (1940)

175. KENSLER, C. J., SUGUIRA, K., AND RHOADS, C. P., *Science, 91, 623 (1940)

176. SYDENSTRICKER, V. P., SEBRELL, W. H., CLECKLEY, H. M., AND KRUSE, H. D., *J. Am. Med. Assoc., 114, 2437 (1940)

177. KRUSE, H. D., SYDENSTRICKER, V. P., SEBRELL, W. H., AND CLECKLEY, H. M., *U.S. Pub. Health Repts., 55, 157 (1940)

178. BESSEY, O. A., AND WOLBACH, S. B., *J. Exptl. Med., 69, 1 (1939)

179. POCK-STEEN, P. H., *Geneeskund. Tijdschr. Nederland.-Indië, 78, 1986 (1939)

180. JOHNSON, L. V., AND ECKARDT, R. E., *Arch. Ophthalmol. (Chicago), 23, 899 (1940)

181. TOPPING, M. C., AND KNOEFEL, A. F., *J. Am. Med. Assoc., 114, 2102 (1940)

182. KLEIN, J. R., AND KOHN, H. I., *J. Biol. Chem., 136, 177 (1940)

183. OCHOA, S., AND ROSSITER, R. J., *Biochem. J., 33, 2008 (1939)

184. SUBRAHMANYAN, V., GREEN, D. E., AND GORDON, A. H., *Nature, 144, 1016 (1939)

185. LASZT, L., AND VERZAR, F., *Arch. ges. Physiol. (Pflügers), 239, 653 (1937)

186. BRUCE, H. M., AND WIEN, R., *J. Physiol., 98, 375 (1940)

187. NELSON, D., *Am. J. Physiol., 129, P 429 (1940)

188. FERREBEE, J. W., *J. Biol. Chem., 136, 719 (1940)

PYRIDOXIN

192. SCUDI, J. V., KOONES, H. F., AND KERESZTESY, J. C., *Am. J. Physiol., 129, P 459 (1940)

193. SCUDI, J. V., BASTEDO, W. A., AND WEBB, T. J., *J. Biol. Chem., 136, 399 (1940)

194. SWAMINATHAN, M., *Nature, 145, 780 (1940)

195. FOUTS, P. J., HELMER, O. M., AND LEPKOVSKY, S., *Am. J. Med. Sci., 199, 163 (1940); *J. Nutrition, 19, 393 (1940)

196. BORSON, H. J., AND METTIER, S. R., *Proc. Soc. Exptl. Biol. Med., 43, 429 (1940)

197. Döllken, H., *Klin. Wochschr.*, **19**, 220 (1940)
198. Reedman, E. J., Sampson, W. L., and Unna, K., *Proc. Soc. Exptl. Biol. Med.*, **43**, 112 (1940)
199. Unna, K., *Proc. Soc. Exptl. Biol. Med.*, **43**, 122 (1940)
200. Chick, H., Macrae, T. F., and Worden, A. N., *Biochem. J.*, **34**, 580 (1940)
201. Weigand, C. G., Eckler, C. R., and Chen, K. K., *Proc. Soc. Exptl. Biol. Med.*, **44**, 147 (1940)
202. McElroy, L. W., and Goss, H., *Proc. Soc. Exptl. Biol. Med.*, **45**, 717 (1940)
203. Chick, H., El Sadr, M. M., and Worden, A. N., *Biochem. J.*, **34**, 595 (1940)
204. Schneider, H. A., Steenbock, H., and Platz, B. R., *J. Biol. Chem.*, **132**, 539 (1940)
205. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **82**, 345 (1929)
206. Tange, U., *Bull. Agr. Chem. Soc. Japan*, **16**, 21 (1940)
207. Salmon, W. D., *J. Biol. Chem.*, **133**, 83 (1940)
208. Supplee, G. C., Bender, R. C., and Kahlenberg, O. J., *J. Nutrition*, **20**, 109 (1940)
209. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940)
210. Emerson, G. A., and Evans, H. M., *Am. J. Physiol.*, **129**, P 352 (1940)
211. Covey, S., and Forbes, J. C., *Virginia Med. Monthly*, **67**, 414 (1940)
212. Unna, K., and Molitor, H., *Am. J. Physiol.*, **129**, P 483 (1940)
213. Unna, K., and Antopol, W., *Proc. Soc. Exptl. Biol. Med.*, **43**, 116 (1940)
214. Ashburn, L. L., *U.S. Pub. Health Repts.*, **55**, 1337 (1940)
215. Scudi, J. V., Koones, H. F., and Keresztesy, J. C., *Proc. Soc. Exptl. Biol. Med.*, **43**, 118 (1940)
216. Scudi, J. V., Unna, K., and Antopol, W., *J. Biol. Chem.*, **135**, 371 (1940)
217. Spies, T. D., Ladisch, R. K., and Bean, W. B., *J. Am. Med. Assoc.*, **115**, 839 (1940)
218. Smith, S. G., and Martin, D. W., *Proc. Soc. Exptl. Biol. Med.*, **43**, 660 (1940)
219. Sebrell, W. H., and Butler, R. E., *U.S. Pub. Health Repts.*, **54**, 2121 (1939)
220. Vilter, R. W., Schiro, H. S., and Spies, T. D., *Nature*, **145**, 388 (1940)
221. Antopol, W., and Schotland, C. E., *J. Am. Med. Assoc.*, **114**, 1058 (1940)
222. Vilter, R. W., Aring, C. D., and Spies, T. D., *J. Am. Med. Assoc.*, **115**, 209 (1940)
223. Pehl, W., *Z. Kinderheilk.*, **61**, 613 (1940)
224. Kock, R., Lozner, E. L., and Meiklejohn, A. P., *Proc. Soc. Exptl. Biol. Med.*, **43**, 97 (1940)
225. Jolliffe, N., *J. Am. Dietet. Assoc.*, **17**, 5 (1941)
226. Spies, T. D., Hightower, D. P., and Hubbard, L. H., *J. Am. Med. Assoc.*, **115**, 292 (1940)
227. Subbarow, Y., and Trager, W., *J. Gen. Physiol.*, **23**, 561 (1940)

228. WILLIAMS, R. J., EAKIN, R. E., AND SNELL, E. E., *J. Am. Chem. Soc.,* **62,** 1204 (1940)

229. WOOLLEY, D. W., AND HUTCHINGS, B. L., *J. Bact.,* **39,** 287 (1940)

230. McILWAIN, H., *Brit. J. Exptl. Path.,* **21,** 25 (1940)

231. VILTER, S. P., AND SPIES, T. D., *Science,* **91,** 200 (1940)

232. HITCHCOCK, A. E., AND ZIMMERMAN, P. W., *Contrib. Boyce Thompson Inst.,* **11,** 143 (1940)

NICOTINIC ACID

233. HARRIS, L. J., AND RAYMOND, W. D., *Biochem. J.,* **33,** 2037 (1939)

234. KODICEK, E., *Biochem. J.,* **34,** 712 (1940)

235. MELNICK, D., AND FIELD, JR., H., *J. Biol. Chem.,* **134,** 1 (1940)

236. MELNICK, D., AND FIELD, JR., H., *J. Biol. Chem.,* **135,** 53 (1940)

237. MELNICK, D., ROBINSON, W. D., AND FIELD, JR., H., *J. Biol. Chem.,* **136,** 131 (1940)

238. MELNICK, D., ROBINSON, W. D., AND FIELD, JR., H., *J. Biol. Chem.,* **136,** 145 (1940)

239. ROSENBLUM, L. A., AND JOLLIFFE, N., *J. Biol. Chem.,* **134,** 137 (1940)

240. BANDIER, E., AND HALD, J., *Biochem. J.,* **33,** 264 (1939)

241. PERLZWEIG, W. A., LEVY, E. D., AND SARETT, H. P., *J. Biol. Chem.,* **136,** 729 (1940)

242. SCHINDEL, L., *J. Lab. Clin. Med.,* **25,** 515 (1940)

243. KODICEK, E., *Biochem. J.,* **34,** 724 (1940)

244. MELNICK, D., ROBINSON, W. D., AND FIELD, JR., H., *J. Biol. Chem.,* **136,** 157 (1940)

245. GUIMARÃES VILLELA, G., *O. Hospital,* **17,** 431 (1940)

246. QUERIDO, A., ALBEAUX-FERNET, M., AND LWOFF, A., *Compt. rend. soc. biol.,* **131,** 182 (1939)

247. WAISMAN, H. A., MICKELSEN, O., McKIBBIN, J. M., AND ELVEHJEM, C. A., *J. Nutrition,* **19,** 483 (1940)

248. AYKROYD, W. R., AND SWAMINATHAN, M., *Indian J. Med. Research,* **27,** 667 (1940)

249. AYKROYD, W. R., *Nature,* **145,** 522 (1940)

250. KRINGSTAD, H., AND THORESEN, F., *Tids. Hermetikind.,* **26,** 113 (1940)

251. PITTMAN, M., AND FRASER, H. F., *U.S. Pub. Health Repts.,* **55,** 915 (1940)

252. AXELROD, A. E., GORDON, E. S., AND ELVEHJEM, C. A., *Am. J. Med. Sci.,* **199,** 697 (1940)

253. KOHN, H. I., *Am. J. Physiol.,* **129,** P 378 (1940)

254. DANN, W. J., AND KOHN, H. I., *J. Biol. Chem.,* **136,** 435 (1940)

255. KOHN, H. I., *Biochem. J.,* **32,** 2075 (1938)

256. SHOURIE, K. L., AND SWAMINATHAN, M., *Indian J. Med. Research,* **27,** 679 (1940)

257. DANN, W. J., KOHN, H. I., AND HANDLER, P., *J. Nutrition,* **20,** 477 (1940)

258. VILTER, R. W., AND SPIES, T. D., *Lancet*, **II**, 423 (1939)
259. BILLS, C. E., McDONALD, F. G., AND SPIES, T. D., *Southern Med. J.*, **32**, 793 (1939)
260. SMITH, D. T., MARGOLIS, G., AND MARGOLIS, L. H., *J. Pharmacol.*, **68**, 458 (1940)
261. BOYD, E. M., AND FORDE, J. D., *J. Pharmacol.*, **70**, 279 (1940)
262. BEAN, W. B., AND SPIES, T. D., *Am. Heart J.*, **20**, 62 (1940)
263. POPKIN, R. J., *Am. Heart J.*, **18**, 697 (1939)
264. FIELD, JR., H., AND ROBINSON, W. D., *Am. J. Med. Sci.*, **199**, 275 (1940)
265. ABRAMSON, D. I., KATZENSTEIN, K. H., AND SENIOR, F. A., *Am. J. Med. Sci.*, **200**, 96 (1940)
266. DAVIS, G. K., FREEMAN, V. A., AND MADSEN, L. L., *Mich. Agr. Expt. Sta., Tech. Bull.*, No. **170**, 23 (1940)
267. WINEGAR, A. H., PEARSON, P. B., AND SCHMIDT, H., *Science*, **91**, 508 (1940)
268. PEARSON, P. B., WINEGAR, A. H., AND SCHMIDT, H., *J. Nutrition*, **20**, 551 (1940)
269. KOHN, H. I., AND BERNHEIM, F., *J. Clin. Investigation*, **18**, 585 (1939)
270. DORFMAN, A., KOSER, S. A., NORWITT, M. A., BERKMAN, S., AND SAUNDERS, F., *Proc. Soc. Exptl. Biol. Med.*, **43**, 434 (1940)
271. KOSER, S. A., DORFMAN, A., AND SAUNDERS, F., *Proc. Soc. Exptl. Biol. Med.*, **43**, 391 (1940)
272. PELCZAR, JR., M. J., AND PORTER, J. R., *Proc. Soc. Exptl. Biol. Med.*, **43**, 151 (1940)
273. VILTER, R. W., BEAN, W. B., RUEGSEGGER, J. M., AND SPIES, T. D., *J. Lab. Clin. Med.*, **25**, 897 (1940)
274. ELVEHJEM, C. A., *Physiol. Rev.*, **20**, 249 (1940)
275. ELVEHJEM, C. A., *Bull. N.Y. Acad. Med.*, **16**, 173 (1940)
276. ROBERTSON, D. S., *Edinburgh Med. J.*, **47**, 81 (1940)
277. BROWNER, J. N., AND BROWNER, A. F., *J. Med. Assoc. Georgia*, **29**, 264 (1940)
278. CAMPBELL, B., *Lancet*, **I**, 738 (1940)
279. MORRIS, H. H., HWANG, H. S., AND KUO, P. T., *Chinese Med. J.*, **57**, 427 (1940)
280. MUSICK, V. H., *J. Oklahoma Med. Assoc.*, **33**, 13 (1940)
281. SALVESEN, O., *Nord. Med.*, **5**, 279 (1940)
282. GOODALL, J. W. D., *Indian Med. Gaz.*, **75**, 147 (1940)
283. MOORE, M. T., *Arch. Internal Med.*, **65**, 1 (1940)
284. HARRIS, H. E., AND MOORE, JR., P. M., *Med. Clinics North Am.*, **24**, 533 (1940)
285. DOUGHTY, J. F., *J. Am. Med. Assoc.*, **114**, 756 (1940)
286. DETWEILER, H. K., KINSEY, H. I., BROWN, W. H., AND FEASBY, U. R., *Arch. Internal Med.*, **65**, 1144 (1940)
287. SAPHIR, W., *Am. J. Digestive Diseases*, **7**, 298 (1940)
288. TEASLEY, H. E., *J. Med. Assoc. Georgia*, **29**, 413 (1940)
289. JOLLIFFE, N., BOWMAN, K. M., ROSENBLUM, L. A., AND FEIN, H. D., *J. Am. Med. Assoc.*, **114**, 307 (1940)

290. KING, J. P., *Lancet,* **II,** 32 (1940)
291. SIEDEK, H., AND REUSS, L., *Wien. klin. Wochschr.,* **52,** 432 (1939)

PANTOTHENIC ACID

292. WILLIAMS, R. J., AND BRADWAY, E., *J. Am. Chem. Soc.,* **53,** 783 (1931)
293. WILLIAMS, R. J., LYMAN, C. M., GOODYEAR, G. H., TRUESDAIL, J. H., AND HOLADAY, D., *J. Am. Chem. Soc.,* **55,** 2912 (1933)
294. WILLIAMS, R. J., AND ROHRMANN, E., *J. Am. Chem. Soc.,* **58,** 695 (1936)
295. WILLIAMS, R. J., WEINSTOCK, JR., H. H., ROHRMANN, E., TRUESDAIL, J. H., MITCHELL, H. K., AND MEYER, C. E., *J. Am. Chem. Soc.,* **61,** 454 (1939)
296. MITCHELL, H. K., WEINSTOCK, JR., H. H., SNELL, E. E., STANBERY, S. R., AND WILLIAMS, R. J., *J. Am. Chem. Soc.,* **62,** 1776 (1940)
297. STILLER, E. T., KERESZTESY, J. C., AND FINKELSTEIN, J., *J. Am. Chem. Soc.,* **62,** 1779 (1940)
298. SNELL, E. E., STRONG, F. M., PETERSON, W. H., *J. Bact.,* **38,** 293 (1939)
299. WILLIAMS, R. J., MITCHELL, H. K., WEINSTOCK, JR., H. H., AND SNELL, E. E., *J. Am. Chem. Soc.,* **62,** 1784 (1940)
300. WOOLLEY, D. W., WAISMAN, H. A., AND ELVEHJEM, C. A., *J. Am. Chem. Soc.,* **61,** 977 (1939)
301. STILLER, E. T., HARRIS, S. A., FINKELSTEIN, J., KERESZTESY, J. C., AND FOLKERS, K., *J. Am. Chem. Soc.,* **62,** 1785 (1940)
302. BABCOCK, JR., S. H., AND JUKES, T. H., *J. Am. Chem. Soc.,* **62,** 1628 (1940)
303. REICHSTEIN, T., AND GRÜSSNER, A., *Helv. Chim. Acta,* **23,** 650 (1940)
304. WOOLLEY, D. W., *J. Am. Chem. Soc.,* **62,** 2251 (1940)
305. WOOLLEY, D. W., *J. Biol. Chem.,* **134,** 461 (1940)
306. WEINSTOCK, JR., H. H., MAY, E. L., ARNOLD, A., AND PRICE, D., *J. Biol. Chem.,* **135,** 343 (1940)
307. MITCHELL, H. K., SNELL, E. E., AND WILLIAMS, R. J., *J. Am. Chem. Soc.,* **62,** 1791 (1940)
308. ZSCHIESCHE, E., AND MITCHELL, H. K., *Proc. Soc. Exptl. Biol. Med.,* **45,** 565 (1940)
309. UNNA, K., AND GRESLIN, J., *Proc. Soc. Exptl. Biol. Med.,* **45,** 311 (1940)
310. JUKES, T. H., *J. Biol. Chem.,* **129,** 225 (1939)
311. SNELL, E. E., PENNINGTON, D., AND WILLIAMS, R. J., *J. Biol. Chem.,* **135,** 213 (1940)
312. PENNINGTON, D., SNELL, E. E., AND WILLIAMS, R. J., *J. Biol. Chem.,* **132,** 65 (1940)
313. DIMICK, M. K., AND LEPP, A., *J. Nutrition,* **20,** 413 (1940)
314. BAUERNFEIND, J. C., AND NORRIS, L. C., *J. Nutrition,* **18,** 579 (1939)
315. PHILLIPS, P. H., AND ENGEL, R. W., *J. Nutrition,* **18,** 227 (1939)
316. WOOLLEY, D. W., *Proc. Soc. Exptl. Biol. Med.,* **43,** 352 (1940)
317. BLACK, S., FROST, D. V., AND ELVEHJEM, C. A., *J. Biol. Chem.,* **132,** 65 (1940)
318. GYÖRGY, P., POLING, C. E., AND SUBBAROW, Y., *J. Biol. Chem.,* **132,** 789 (1940)

319. György, P., and Poling, C. E., *Science*, **92**, 202 (1940)
320. György, P., and Poling, C. E., *Proc. Soc. Exptl. Biol. Med.*, **45**, 773 (1940)
321. Vigneaud, V. du, Melville, D. B., György, P., and Rose, C. S., *Science*, **92**, 62 (1940)
322. Hegsted, D. M., Oleson, J. J., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, **20**, 627 (1940)
323. Robbins, W. J., and Hamner, K. C., *Botan. Gaz.*, **101**, 912 (1940)
324. Schopfer, W. H., *Arch. Mikrobiol.*, **6**, 510 (1935)
325. Mohammad, A., Emerson, O. H., Emerson, G. A., and Evans, H. M., *J. Biol. Chem.*, **133**, 17 (1940)
326. Nielsen, E., Oleson, J. J., and Elvehjem, C. A., *J. Biol. Chem.*, **133**, 637 (1940)
327. Morgan, A. F., and Simms, H. D., *J. Nutrition*, **19**, 233 (1940)
328. Morgan, A. F., and Simms, H. D., *Science*, **89**, 565 (1939)
329. Morgan, A. F., and Simms, H. D., *J. Nutrition*, **20**, 627 (1940)
330. Lunde, G., and Kringstad, H., *J. Nutrition*, **19**, 321 (1940)
331. Unna, K., and Sampson, W. L., *Proc. Soc. Exptl. Biol. Med.*, **45**, 309 (1940)
332. Williams, R. R., *Science*, **92**, 561 (1940)
333. Free, A. H., *Proc. Soc. Exptl. Biol. Med.*, **44**, 371 (1940)
334. Gorter, F. J., *Nederland.-indische bl. diergeneeskunde*, **51**, 347 (1939)
335. Stirn, F. E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **109**, 347 (1935)
336. Ansbacher, S., *Proc. Soc. Exptl. Biol. Med.*, **44**, 248 (1940)
337. Berkman, S., Saunders, F., and Koser, S. A., *Proc. Soc. Exptl. Biol. Med.*, **44**, 68 (1940)
338. Rane, L., and Subbarow, Y., *J. Biol. Chem.*, **134**, 455 (1940)
339. Pelczar, Jr., M. J., and Porter, J. R., *Proc. Soc. Exptl. Biol. Med.*, **43**, 151 (1940)
340. Happold, F. C., *Proc. Soc. Exptl. Biol. Med.*, **43**, 412 (1940)
341. Richardson, L. R., and Hogan, A. G., *Proc. Soc. Exptl. Biol. Med.*, **44**, 583 (1940)
342. Unna, K., *J. Nutrition*, **20**, 565 (1940)
343. Chick, H., Macrae, T. F., Martin, A. J. P., and Martin, C. J., *Biochem. J.*, **32**, 2207 (1938)
344. Wintrobe, M. M., Mitchell, D. M., and Kolb, L. C., *J. Exptl. Med.*, **68**, 207 (1938)
345. McKibbin, J. M., Madden, R. J., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, **128**, 102 (1939)
346. Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition*, **16**, 197 (1938)
347. McKibbin, J. M., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, **130**, 365 (1940)
348. Lunde, G., and Kringstad, H., *Naturwissenschaften*, **27**, 255 (1939)
349. Daft, F. S., and Sebrell, W. H., *U.S. Pub. Health Repts.*, **54**, 2247 (1939)

350. NELSON, A. A., *U.S. Pub. Health Repts.,* 54, 2250 (1939)
351. DAFT, F. S., SEBRELL, W. H., BABCOCK, JR., S. H., AND JUKES, T. H., *U.S. Pub. Health Repts.,* 55, 1333 (1940)
352. MILLS, R. C., SHAW, J. H., ELVEHJEM, C. A., AND PHILLIPS, P. H., *Proc. Soc. Exptl. Biol. Med.,* 45, 482 (1940)
353. SALMON, W. D., AND ENGEL, R. W., *Proc. Soc. Exptl. Biol. Med.,* 45, 621 (1940)
354. STANBERY, S. R., SNELL, E. E., AND SPIES, T. D., *J. Biol. Chem.,* 135, 353 (1940)
355. SURE, B., *J. Nutrition,* 19, 57 (1940)
356. NAKAHARA, W., INUKAI, F., AND UGAMI, S., *Science,* 91, 431 (1940)
357. NAKAHARA, W., INUKAI, F., AND UGAMI, S., *Sci. Papers Inst. Phys. Chem. Research (Tokyo),* 36, 327 (1939)
358. NAKAHARA, W., INUKAI, F., AND UGAMI, S., *Science,* 93, 39 (1941)
359. JUKES, T. H., *Proc. Soc. Exptl. Biol. Med.,* 45, 625 (1940)

OTHER B FACTORS

360. KÖGL, F., AND TÖNNIS, B., *Z. physiol. Chem.,* 242, 43 (1936)
361. WEST, P. M., AND WILSON, P. W., *Science,* 89, 608 (1939)
362. NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., *Naturwissenschaften,* 27, 389 (1939)
363. ALLISON, F. E., HOOVER, S. R., AND BURK, D., *Science,* 78, 217 (1933)
364. GYÖRGY, P., MELVILLE, D. B., BURK, D., AND VIGNEAUD, V. DU, *Science,* 91, 243 (1940)
365. SNELL, E. E., EAKIN, R. E., AND WILLIAMS, R. J., *J. Am. Chem. Soc.,* 62, 175 (1940)
366. GYÖRGY, P., ROSE, C. S., HOFMANN, K., MELVILLE, D. B., AND VIGNEAUD, V. DU, *Science,* 92, 609 (1940)
367. EAKIN, R. E., McKINLEY, W. A., AND WILLIAMS, R. J., *Science,* 92, 224 (1940)
368. EAKIN, R. E., SNELL, E. E., AND WILLIAMS, R. J., *J. Am. Chem. Soc.,* 62, 1204 (1940)
369. BECHDEL, S. J., ECKLES, C. H., AND PALMER, L. S., *J. Dairy Sci.,* 9, 409 (1928)
370. McELROY, L. W., AND GOSS, H., *J. Biol. Chem.,* 130, 437 (1939)
371. McELROY, L. W., AND GOSS, H., *J. Nutrition,* 20, 527 (1940)
372. McELROY, L. W., AND GOSS, H., *Proc. Soc. Exptl. Biol. Med.,* 45, 296 (1940)
373. McELROY, L. W., AND GOSS, H., *J. Nutrition,* 20, 541 (1940)
374. WEGNER, M. W., BOOTH, A. N., ELVEHJEM, C. A., AND HART, E. B., *Proc. Soc. Exptl. Biol. Med.,* 45, 769 (1940)
375. KNIGHT, C. A., DUTCHER, R. A., GUERRANT, N. B., AND BECHDEL, S. J., *Proc. Soc. Exptl. Biol. Med.,* 44, 90 (1940)
376. RANDLE, S. B., SOBER, H. A., AND KOHLER, G. O., *J. Nutrition,* 20, 459 (1940)

377. Winegar, A. H., and Pearson, P. B., *Proc. Am. Soc. Animal Production*, **32**, 384 (1939)

378. Woolley, D. W., *J. Biol. Chem.*, **136**, 113 (1940)

379. Woolley, D. W., *Science*, **92**, 384 (1940)

380. Norris, E. R., and Hauschildt, J., *Science*, **92**, 316 (1940)

381. Hogan, A. G., and Parrott, E. M., *J. Biol. Chem.*, **132**, 507 (1940)

382. Hogan, A. G., Richardson, L. R., Johnson, P. E., and Nisbet, R. N., *J. Nutrition*, **20**, 203 (1940)

383. Dameshek, W., and Myerson, P. G., *Am. J. Med. Sci.*, **119**, 518 (1940)

384. Hegsted, D. W., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *Poultry Sci.*, **19**, 167 (1940)

385. Stokstad, E. L. R., Manning, P. D. V., and Rogers, R. E., *Poultry Sci.*, **19**, 412 (1940)

386. Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, **134**, 213, 465 (1940)

387. Stokstad, E. L. R., Manning, P. D. V., and Rogers, R. E., *J. Biol. Chem.*, **132**, 463 (1940)

388. Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, **135**, 313 (1940)

389. Schumacher, A. E., and Heuser, G. F., *Poultry Sci.*, **19**, 315 (1940)

390. Jukes, T. H., *J. Nutrition*, **20**, 445 (1940) ; *J. Biol. Chem.*, **134**, 789 (1940)

391. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **133**, 627 (1940)

392. Griffith, W. H., *J. Nutrition*, **19**, 437 (1940)

393. Curtis, A. C., Newburgh, L. H., and Thomas, F. H., *Arch. Internal Med.*, **39**, 817, 828 (1927)

394. Griffith, W. H., *J. Biol. Chem.*, **133**, 639 (1940)

395. Vigneaud, V. du, Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939); Chandler, J. P., and Vigneaud, V. du, *J. Biol. Chem.*, **135**, 223 (1940)

396. Vigneaud, V. du, Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940)

397. Sure, B., *J. Nutrition*, **19**, 71 (1940)

398. György, P., and Goldblatt, H., *J. Exptl. Med.*, **72**, 1 (1940)

399. Daggs, R. G., *Am. J. Obstet. Gynecol.*, **40**, 457 (1940)

400. Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **127**, 211 (1939)

401. Perlman, I., Stillman, N., and Chaikoff, I. L., *J. Biol. Chem.*, **133**, 651 (1940)

VITAMIN C

402. Ludden, J. B., and Wright, I., *Arch. Internal Med.*, **65**, 151 (1940)

403. Friedman, G. J., Sherry, S., and Ralli, E. P., *J. Clin. Investigation*, **19**, 685 (1940)

404. Sherry, S., Friedman, G. J., Paley, K., Berkman, J., and Ralli, E. P., *Am. J. Physiol.*, **130**, 276 (1940)

405. Pillay, A. P., *Indian Med. Gaz.*, **75**, 91 (1940)

406. GREENBERG, L. D., AND RINEHART, J. F., *J. Lab. Clin. Med.*, **25**, 1288 (1940)
407. KASSAN, R. J., AND ROE, J. H., *J. Biol. Chem.*, **133**, 579 (1940)
408. HEINEMANN, M., AND HALD, P. M., *J. Clin. Investigation*, **19**, 469 (1940)
409. BUTLER, A. M., AND CUSHMAN, M., *J. Clin. Investigation*, **19**, 459 (1940)
410. MINDLIN, R. L., *J. Pediat.*, **16**, 275 (1940)
411. KAJDI, L., LIGHT, J., AND KAJDI, C., *J. Pediat.*, **15**, 197 (1940)
412. AHLBORG, N. G., AND BRANTE, G., *Acta Med. Scand.*, **104**, 527 (1940)
413. RAPAPORT, H. G., MILLER, S. H., AND SICULAR, A., *J. Pediat.*, **16**, 624 (1940)
414. DIFS, H., *Acta Med. Scand.*, Suppl., **110**, 167 (1940)
415. BANERJEE, S., GUHA, B. C., AND BASAK, K. C., *Indian Med. Gaz.*, **75**, 468 (1940)
416. BAKHSH, I., KOCHLAR, B. D., AND MALIK, A. Q., *Indian J. Med. Research*, **27**, 695 (1940)
417. BECK, H., AND KRIEGER, F. H., *Deut. med. Wochschr.*, **65**, 1336 (1939)
418. LEMMEL, G., *Deut. Arch. klin. Med.*, **183**, 277 (1939)
419. TODHUNTER, E. N., AND ROBBINS, R. C., *J. Nutrition*, **19**, 263 (1940)
420. RALLI, E. P., FRIEDMAN, G. J., AND SHERRY, S., *J. Clin. Investigation*, **18**, 705 (1939)
421. CHEN, K. C., YU, T. F., LIU, S. H., AND CHU, H. I., *Chinese J. Physiol.*, **15**, 119 (1940)
422. CRANDON, J. H., AND LUND, C. C., *New Engl. J. Med.*, **222**, 748 (1940)
423. CRANDON, J. H., LUND, C. C., AND DILL, D. B., *New Engl. J. Med.*, **223**, 353 (1940)
424. RIETSCHEL, H., AND MENSCHING, J., *Klin. Wochschr.*, **18**, 273 (1939)
425. BARTLETT, M. K., JONES, C. M., AND RYAN, A. E., *Ann. Surg.*, **111**, 1 (1940)
426. ABT, A. F., FARMER, C. J., TOPPER, Y. J., *Proc. Soc. Exptl. Biol. Med.*, **43**, 24 (1940)
427. MOURIQUAND, G., *Ann. méd.*, **46**, 249 (1940); MOURIQUAND, G., DAUVERGNE, M., TÊTE, H., AND EDEL, V., *Compt. rend.*, **210**, 515 (1940); JAQUES, R. H., *J. Bone Joint Surg.*, **22**, 324 (1940)
428. KAPLAN, A., AND ZONNIS, M. E., *Am. Rev. Tuberc.*, **42**, 667 (1940)
429. OSBORNE, T. W. B., AND GEAR, J. H. S., *Nature*, **145**, 974 (1940)
430. STEINBACH, M. M., AND DUCA, C. J., *Proc. Soc. Exptl. Biol. Med.*, **44**, 133 (1940)
431. ERWIN, G. S., WRIGHT, R., AND DOHERTY, C. J., *Brit. Med. J.*, **1**, 688 (1940)
432. FIELD, JR., H., ROBINSON, W. D., AND MELNICK, D., *Ann. Internal Med.*, **14**, 588 (1940)
433. HAUSBERGER, F. X., AND NEUENSCHWANDER-LEMME, N., *Klin. Wochschr.*, **18**, 1119 (1939)
434. GORTZ, G., *Acta Paediat.*, **27**, 429 (1940)
435. KALK, H., *Leut. med. Wochschr.*, **65**, 1624 (1940)
436. MERRITT, H. H., AND FOSTER, A., *Am. J. Med. Sci.*, **200**, 541 (1940)
437. JENOVESE, J. F., OSTERBERG, A. E., AND RYNEARSON, E. H., *Proc. Soc. Exptl. Biol. Med.*, **44**, 335 (1940)

438. Schätzlein, C., and Fox-Timmling, E., *Z. Untersuch. Lebensm.*, **79,** 157 (1940)
439. French, R. B., and Abbott, O. D., *J. Nutrition*, **19,** 223 (1940); Metcalfe, E., Rehm, P., and Winters, J., *Food Research*, **5,** 233 (1940)
440. Richmond, M. S., Satterfield, G. H., Grinnells, C. D., and Dann, W. J., *J. Nutrition*, **20,** 99 (1940)
441. Woessner, W. W., Elvehjem, C. A., and Schuette, H. A., *J. Nutrition*, **20,** 327 (1940)
442. Holmes, A. D., Tripp, F., Woelffer, E. A., and Satterfield, G. H., *Food Research*, **5,** 263 (1940); *Am. J. Diseases Children*, **60,** 1025 (1940)
443. Satterfield, G. H., and Yarbrough, M., *Food Research*, **5,** 241 (1940)
444. Burrell, R. C., Brown, H. D., and Ebright, V. R., *Food Research*, **5,** 247 (1940)
445. Mayfield, H. L., and Richardson, J. E., *Food Research*, **5,** 361 (1940)
446. Floyd, W. W., and Fraps, G. S., *Food Research*, **5,** 33 (1940)
447. McIntosh, J. A., and Tressler, D. K., *J. Home Econ.*, **32,** 692 (1940)
448. Clayton, M. M., and Folsom, M. T., *J. Home Econ.*, **32,** 390 (1940)
449. Todhunter, E. N., and Fatzer, A. S., *J. Nutrition*, **19,** 121 (1940)
450. Schümmer, H., *Biochem. Z.*, **304,** 1 (1940); Schrens, H. T., and Schümmer, H., *Biochem. Z.*, **304,** 18 (1940)
451. Ramasarma, G. B., Datta, N. C., and Doctor, N. S., *Enzymologia*, **8,** 108 (1940)
452. Houston, J., Kon, S. K., and Thompson, S. Y., *J. Dairy Research*, **10,** 471 (1939); Henderson, J. L., Foord, D. C., and Roadhouse, C. L., *Food Research*, **5,** 153 (1940)
453. Woessner, W. W., Weckel, K. G., and Schuette, H. A., *J. Dairy Sci.*, **23,** 1131 (1940)
454. Gould, I. A., *J. Dairy Sci.*, **23,** 991 (1940)
455. Swanson, A. M., and Sommer, H. H., *J. Dairy Sci.*, **23,** 597 (1940)
456. Hand, D. B., Guthrie, E. S., and Sharp, P. F., *Science*, **87,** 439 (1938)
457. Sealock, R. R., and Silberstein, H. E., *Science*, **90,** 517 (1939)
458. Levine, S. Z., Marples, E., and Gordon, H. H., *Science*, **90,** 620 (1939)
459. Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, **135,** 251 (1940)
460. Sealock, R. R., Galdston, M., and Steele, J. M., *Proc. Soc. Exptl. Biol. Med.*, **44,** 580 (1940)
461. Rothman, S., *Proc. Soc. Exptl. Biol. Med.*, **45,** 52 (1940)
462. Ball, E. G., *J. Biol. Chem.*, **134,** 177 (1940)
463. Edlbacher, S., and Segesser, A. v., *Biochem. Z.*, **290,** 370 (1937)
464. Greenblatt, I. J., and Pecker, A., *J. Biol. Chem.*, **134,** 341 (1940)
465. Milhorat, A. T., Bartels, W. E., and Toscani, V., *Proc. Soc. Exptl. Biol. Med.*, **45,** 394 (1940)
466. Milhorat, A. T., Hardy, J. D., Bartels, W. E., and Toscani, V., *Proc. Soc. Exptl. Biol. Med.*, **45,** 397 (1940)
467. Ralli, E. P., and Sherry, S., *Proc. Soc. Exptl. Biol. Med.*, **43,** 669 (1940)
468. Thaddea, S., and Scharsach, F., *Biochem. Z.*, **305,** 109 (1940)
469. Leblond, C. P., and Chamorro, A., *Compt. rend.*, **133,** 71 (1940)
470. Holtz, P., and Walter, H., *Klin. Wochschr.*, **19,** 136 (1940); *Z. physiol. Chem.*, **263,** 187 (1940); Holtz, P., and Reischel, C., *Klin. Wochschr.*, **19,** 461 (1940)

FAT-SOLUBLE VITAMINS[1]

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VITAMIN A

Chemistry.—A study of elimination curves has led to the belief that vitamins A_1 and A_2 differ not by an additional CH_2 group in A_2 but by an additional conjugate double bond (1). Like A_1, A_2 can be "cyclized" by dry alcoholic hydrogen chloride (2); since the product is more strongly adsorbed by alumina than is cyclized A_1, this difference may prove to be a means of separating the two forms in a natural mixture. Because of the spontaneous isomerization of β-carotene to pseudo α-carotene (3), its solutions should be stored at 0° and should be adsorbed by alumina just prior to use. The biologically inactive isocarotene is believed to be dehydro-β-carotene (4). Repetition of the original synthetic procedure of Kuhn & Morris has revealed (5) that the main product is probably not vitamin A although this may be present. Preparations of A obtained by crystallization are not necessarily purer than those produced by chromatographic analysis (6). Crystalline vitamine-A palmitate was synthesized and examined spectrophotometrically (7).

The so-called carotene oxidase does not exist (8); the oxidation of carotene is probably coupled with that of unsaturated fats. Several studies have confirmed the destructive action of rancid fats on vitamin A (9). Carotene has been isolated from green plant tissue by an improved method (10), and the preparation and keeping qualities of colloidal solutions of vitamin A and the carotenoids have been described (11).

Assay.—Relatively little attention has been given to biological methods of assay (12, 13); much more work has been done on colorimetric and spectrographic methods. The principal difficulty in the

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[1] Of the five hundred papers on the fat-soluble vitamins which have been published since the last review only a portion are here reviewed. The bibliography contains entries available to about December 20, 1940. The relation of all the vitamins to the sex glands has been recently summarized (6a), and the vitamin content (including carotenoids) of foods has appeared in extensive tables (7a).

use of spectrographic methods is to obtain a factor for the conversion of the absorption data ($E_{1\,cm}^{1\%}$ at 328 mμ) to international units (I.U.) of biological activity; factors varying from 1,750 to 3,200 were found for a series of cod-liver oils (14). Spectrographic results may agree more closely than biological assays if the conversion factor is determined daily (15); but the instruments and solutions must be checked frequently and the technique must be rigorously uniform (16).

The colorimetric method also has disadvantages. Other chromogens, and also color inhibitors, must be reckoned with when it is applied to oils (17). The former can be removed by selective solvents or by chromatographic adsorption (18), but the specific action of the adsorbent must first be tested (19). The use of alkali in the presence of carbohydrates may prevent complete extraction of the carotene (20).

A spectrophotometric study showed (21) that color density is not a strictly linear function of the concentration of A or carotene. The proposed (22) conversion factor ($L_{1\,cm}^{1\%}$ 440 mμ to I.U.) for β-carotene was found to be 1,645 and 1,980 in chloroform and Skellysolve, respectively, and for U.S.P. reference cod-liver oil (620 mμ), 3.45. The spectrophotometric assay of A in fortified margarine and in fish liver oils has been studied critically and fully described (23).

A simple photoelectric colorimeter has been described (24), as well as rapid methods for determining carotene and vitamin A in blood (25); in the interpretation of such figures the blood lipids should be taken into account (26). The determination of carotene and allied pigments in dry grass and other leafy materials and their constitution and physiological significance have been reviewed (27, 28).

Occurrence.—Scores of papers have presented the vitamin-A or carotene content of various animal and human foods and the effects of storage, canning, or other treatment. Many of these reports have appeared in food journals and bulletins and the references can be found in various annual indexes. Of particular interest are the distribution of astaxanthin in the animal and plant kingdom (29) and the occurrence of vitamin A and carotenoids in the livers of twenty-one mammals, thirty-six birds, two reptiles, and man (30). There was no relation between the amounts of the two found in food and in the liver. Most livers contained no carotenoid; vitamin A_2 was never found. Aphanin and aphanicin, two new carotenoid pigments having some vitamin-A potency (31), were isolated from a blue fresh-water alga. Doubling the number of chromosomes in pure yellow corn pro-

duced a 40 per cent increase of the carotenoid content, and in white corn, a 19 per cent decrease (32) ; external appearance is not a reliable criterion of the carotenoid content.

With the supplies of Norwegian cod-liver oil cut off by the war, other sources of vitamin A are being sought—among them the liver oils of Argentine, Brazilian, Australian, and Indian fish (33)—as well as further information on the liver and body oils of the halibut and cod, the seal, the shark, and the geelbeck (34).

The carotenoid and vitamin-A content of eggs is closely dependent on dietary supply (35) ; during incubation of eggs the A content declines, although the carotene does not (36). In sheep and cow colostrum A is much more abundant than it is in the later milk (37) ; "light white" casein contains about 1 I.U. of A per gram, which is removable by hot alcohol (38). Roller-process dried milk is not as deficient as originally supposed (39).

Absorption, utilization, and requirements.—The interference of mineral oil with full utilization of carotene and vitamin A has been confirmed by tests on rats and dogs (40). In human subjects the loss may be largely by solution in the oil, and prolonged use should be accompanied by a diet high in vitamin A (41), or the oil should be saturated (at 37°) with carotene or A (42).

Vitamin-A esters are hydrolyzed by intestinal lipases, and during the height of absorption, A exists in the gut wall chiefly as the alcohol (43). When various forms of A were fed, variable proportions were recovered from the liver as esters, most (56 per cent) from U.S.P. oil and least (10 per cent) from β-carotene (44). Since the esters were all similar, selected fatty acids may be used for esterification. The delay following administration of carotene is presumably conditioned by slower absorption and the process of conversion ; this was demonstrable after two hours in the rat (45). Finely emulsified A, given intravenously to rabbits and man, rapidly disappeared from the blood (46) as if the tissues took it up with exceptional speed. In xerophthalmic rats on an almost fat-free diet, carotene was curative when fed in an aqueous colloidal solution (47). In various species subcutaneous and intramuscular injections of A or carotene were less effective than when they were given by mouth (48).

The hedgehog also utilizes carotene less well than it does A, as measured by the increase of A in the liver (49). The concentrations of both in liver and serum varied seasonally, and carotene may favor the onset of hibernation (50).

A study of the absorption of carotene from carrots in man showed their vitamin-A value to be low (51) whereas the absorption of carotene and A from butter was nearly quantitative. Another study of the utilization of the carotene of cooked green peas and spinach (52), measured by the amount of each necessary to maintain normal dark adaptation in adults on a mixed diet, indicated that 47 to 57 I.U. per kilogram of body weight were required in the form of peas and 77 to 101 I.U. in the form of spinach. The vitamin-A value of these vegetables was less well utilized than that of cod-liver oil but better than that of carotene dissolved in cottonseed oil. The addition of fat beyond the 30 per cent supplied by the mixed diet did not increase the utilization. Many variables enter into this evaluation, including digestibility, rate of absorption and conversion, and in particular the final criterion of dark adaptation which is to be discussed later.

The final word has not yet been said on this question of utilization; it is intimately bound up with that of minimum requirements.

Studies of the vitamin-A needs of farm animals (53, 54) and especially of the horse (53) have been made. Nyctolopia and edema appeared in calves when the plasma carotene fell below 0.13 µg. per cc. (55); 11 µg. to 16 µg. of carotene per pound of body weight per day were required, depending on its availability from the sources used, and younger calves required even more (55, 56). The needs of range cattle and dairy cows were determined both as to carotene and A (57). The factor in soybean oil which interferes with the transfer of carotene to the milk fat can be adsorbed from the oil by activated carbon (58).

The requirements of chicks and laying hens have been studied (59) and reviewed (28, 60); the reserves of A in young chicks are small (61). The vitamin-A content of the livers of dogs showed extreme variations (62). The low hemoglobin and red cell count in A-deficient dogs was not due to lack of A (63). The requirements of silver foxes were also the subject of an inquiry (64).

The minimum daily requirements of rats to prevent cornification were 3.8 to 4.6 µg. of A and 15 to 20 µg. of carotene per kg. of body weight (65). On two different basal diets rats responded differently to A and to carotene both as to fecal excretion and liver storage (66); there is need of an adequate standardized basal diet free from such discrepancies, and an approach to such a diet has been made (13). Rats kept in the dark stored vitamin A in the liver at a more rapid rate than those kept in the light (67). There appears to be some storage of A in muscle (68), but the lesions attributed to lack of it

are traceable to simultaneous lack of vitamin E (69). Growth and hepatic storage of A were decreased by intraperitoneal injection of 1,2,4,6-dibenzeneanthracene (70). Cockroaches do not need vitamin A (71).

Several unusual pathological observations were made on animals deprived of vitamin A. An extensive study of pigs demonstrated nerve lesions, gradual muscular in-co-ordination, and paralysis, as well as decrease in visual power (72). Accompanying nyctalopia in calves was an increased cerebrospinal fluid pressure (73). Lack of A is one factor in the production of stomach lesions in rats (74); rats deficient in A also showed degeneration in the funiculus praedorsalis of the medulla (75). Defects in tooth and gingival structure have also been described, and the A requirement appears to increase with age (76). Keratoconus was experimentally produced in young rats by controlled deprivation and subsequent feeding of A (77).

Attempts to demonstrate functional antagonism between vitamin A and the thyroid with pigeons, cats, and rats have given rise to contradictory conclusions, and as yet a specific antagonism remains to be demonstrated (78). In A-deficient rats the gonadotropic hormone content of the anterior hypophysis is increased (79). The tissue changes produced by prolonged partial deprivation of vitamin A were more severe in castrated rats when these were given estrogens (80).

Dark adaptation.—Although night blindness is recognized as one of the first observable symptoms of vitamin-A deprivation in man and animals, all shades of opinion have been expressed regarding the validity of dark adaptation tests as an adequate index to the vitamin-A status and treatment of human subjects.

Since the changes in the early cone response are of smaller magnitude than those in the later rod response it is argued (81) that instruments and techniques designed for measurements in the early rapid stages of dark adaptation are less reliable than those using the later rod response after thirty minutes of darkness. On the other hand since the two (log) thresholds rise in a linear manner during deprivation (82), values for either one, if determined accurately, should be equally significant. In both cases the ability of subjects to co-operate is highly essential and a short learning period may be necessary. When such a practice period preceded each test (83), the results obtained from readings made during a ten-minute dark adaptation period were shown to be correlated with the diet and the vitamin-A status of children and to possess statistical validity. Other dietary

deficiencies as well as metabolic variables may affect the test. The presence of infection, especially respiratory infection, is commonly not taken into adequate account, and the seasonal incidence of dysadaptation may be conditioned as much by this as by diet (83).

It is thus entirely possible that the disconcerting contradictions obtained by the use of the dark adaptation test are due to unknown variables in the subjects tested rather than to differences between instruments used to make the test (84).

Recent experiments on adult human subjects (81, 82, 85, 86) have generally shown that the gradual rise in threshold of vision following deprivation of vitamin A begins immediately, but the extent of temporary restoration by single large doses of A or carotene varies greatly, from 0 to 100 per cent, and this may (82) or may not (81) be correlated with dietary supplements of minerals and some of the other vitamins. The amounts of A and the time interval required for permanent restoration also vary. After a 188-day deprivation period, increasing doses of A or carotene were given daily to the ten subjects investigated (85). A daily dose of 3,000 µg. of β-carotene finally restored normal thresholds between the forty-fifth and the sixty-first day. Smaller amounts of vitamin A sufficed.

A new apparatus and rapid test have been described (87), together with limiting and nonlimiting factors; the results showed a definite correlation with the vitamin-A content of the blood (88) as determined by a somewhat new method. The observation that vitamin A is mobilized into the blood of dogs from its stores in the tissues (liver) by ethyl alcohol (89), probably by sympathicoadrenal stimulation (90), has been further corroborated by the demonstration that the taking of alcohol by human subjects reduced the recovery time in the dark adaptation test (91). Other judgments that have been rendered on this subject include the absence of any correlation between the vitamin A of the blood and biophotometer readings, a correlation of night blindness with diet but not with age, and a relation between poor adaptation and recurrent seasonal keratoconjunctivitis for which the determination of A in serum provided further support (92). Subnormal dark adaptation does not necessarily indicate vitamin-A deficiency (93), for it may be found even with high intake, but good response is not possible with a low intake (94). Biophotometer response and dietary A were correlated in a hundred American families (94) and in French and American children (95).

The adequacy of the vitamin-A supplies in pregnancy were satis-

factorily studied by means of a new adaptation apparatus (96), but single-test determinations of the vitamin-A level were valid only if pathological adaptation times were verified repeatedly over short periods (97). The biophotometric method was found not to be precise for determining vitamin-A deficiency in skin diseases (98).

Based on adaptation studies or on the concentration of A and carotene in the blood, the suggestion is made (99) that liver damage may interfere with the conversion of carotene to vitamin A; the failure of diets rich in A to produce improvement of adaptation in cases of cirrhosis may be due to alteration in the metabolism of A (100). In jaundice the diet is often deficient and there is deficient absorption and storage (101). The poor dark adaptation of juvenile diabetics was improved by A and not by carotene (102). Vitamin A appears not to be important for the respiration of liver tissue (103). The change in dark adaptation produced by lack of oxygen is unlike that produced by a deficiency of A and is unaltered by it (104).

The visual test can thus not be condemned as a measure of vitamin-A intake or status until the variables concerned have been more carefully indicated (105).

Large doses of vitamin A may produce moderate increases in urea clearance (106). Oils containing vitamin A have a protective action on superficial corneal lesions but the action is systemic rather than local (107). Myopia is more commonly found when fats, vitamin A, and carotene are lacking in the diet (108).

Vitamin A is not specifically concerned with resistance to tuberculosis in pigs or in man (109), although a generous allowance is recommended for certain conditions in this disease (110). Carotenoids are not essential for the growth of the paratubercle bacillus (111). The use of vitamin A in therapy has been briefly summarized (112).

Vitamin D

Chemistry.—Except for chemical studies of the biologically inactive dihydrovitamins D_2 and D_3 and photopyrocalciferols (113), no contributions seem to have been made to the purely chemical aspects of antirachitic substances. The superior effectiveness of monochromatic light of wave length 2967 Å in activating 7-dehydrocholesterol supports the belief that this substance is a significant precursor of D in the skin (114).

Assay.—Spectrographic determination of D in oils is unsatisfactory because no relation has been found between extinction coefficient

and D content as determined biologically (115). Vitamin A interferes, and its adsorption with acid clay also removes some D. Uniform values for the absorption of even a single constituent (D_2) have not been obtained (116) largely because of its photolability and the variable exposures required by different types of photometers.

Various color reactions have been proposed for the detection and estimation of vitamin D. That of Tortelli & Jaffe (116a) (bromine in chloroform) is said to have absorption bands suitable for quantitative photocolorimetric determination (117). The yellow color produced by antimony trichloride [Brockmann & Chen (117a)] with D_2 and D_3 varies in intensity with age and sensitivity of the reagent (118), and the method is unsuitable for use with oils containing A or a low concentration of D. These and other difficulties have been largely overcome by the use of a new reagent, antimony trichloride and acetyl chloride in chloroform (119), which produces a yellowish pink color, reaching a maximum intensity in thirty seconds and remaining stable for four to five minutes. Absorption curves for D_2 and D_3 are identical, with the absorption maximum at 500 mμ and $E_{1\ cm.}^{1\%}$ about 1,800. Optical density is directly proportional to the vitamin concentration and as little as 0.2 μg. can be determined accurately. It remains to apply this promising method to natural sources.

The three biological methods of assay (120) involve large individual variations. This is true even of the prophylactic bone ash method (121), the results of which are expressed in definite figures. These variations are much more evident with the line-test and x-ray methods, in which the findings require interpretation. Suggested modifications of these techniques have aimed at quantification of results which can be correlated. With rats as test animals, statistically significant relationships were established (122) between decreasing size of dose and increased distance between diaphysis and epiphysis at interception of medial and lateral condyles; this distance was measured in enlarged radiographs of living animals. Plotted observations checked within 10 per cent of a theoretical curve; litter mates gave best agreement. The Olsson technique was improved (123) and greater accuracy was obtained if the logarithm of the tarsometatarsal distance rather than the actual distance was accepted as a function of dietary D_3; since D_3 is more effective than calciferol in birds, it should be used as a standard of reference in the assay of poultry feeds. A modification of the line test consisted in applying the reagent to 0.5 mm. serial sections of the entire condyles (124); shape

and density of the calcified areas aided in arriving at a calcification index which showed good correlation with dietary D supplements. Gain in weight, bone ash content, and degree of healing gave excellent correlation (125) in twenty-four-day tests on ten-day-old depleted chicks. The width of the epiphyseal cartilage in young rats decreases gradually up to thirty-six days of age, but a D-deficient diet causes a widening of this cartilage which continues for three to four weeks (126).

Since dietary supplements of phosphorus greatly enhance the potency of added D (with diets high in calcium and low in phosphorus) and not all phosphorus compounds have the same enhancement factor (127), it is necessary to compensate for this variable; thus, in the determination of D in milk, the chicks on reference oil should receive an equivalent amount of the solids-not-fat (128) or in the case of other phosphorus-containing foods, their D content should be assayed on an extract after saponification (129). Conversely, a rachitogenic diet low in calcium and high in phosphorus is made more rachitogenic by replacement of the phosphate by oxalate (130). A known amount of D produces better healing on a diet containing 5 per cent of fat than on one almost fat-free (131).

Fluorine in a rachitogenic diet seems to decrease the severity of the rickets but administration of D promotes deposition of atypical bone salt (132); when fluorine is given with D to typically rachitic rats the healing process is inhibited.

Little new information has become available on the manner of action of D. Tests with radioactive phosphorus showed (133) that D has no influence on the entrance of phosphorus into the blood or into the diaphysis, but in metaphysis a positive line test accompanies an increased radioactive phosphorus content within fifty-four to seventy-two hours after administration.

Although D thus has other functions, its favorable influence on the intestinal absorption of calcium and phosphorus is still receiving emphasis. Calciferol decreased the fecal excretion of calcium in nephrectomized rats immediately after thyroparathyroidectomy (134); on a basal rachitogenic ration containing 5 per cent of mineral oil, three times the usual amount of cod-liver oil was required to cure rickets in rats and dogs, and more was needed with 10 per cent of mineral oil (135).

Cereal grains do not contain a specific anticalcifying factor (136); the calcifying power of cereals is dependent primarily upon their

content of calcium and available phosphorus. The availability of phytic acid phosphorus is not as great as that of inorganic phosphorus, even in the presence of D, and is markedly affected by the calcium and especially by the vitamin-D content of the diet (137); the calcium of calcium phytate is as available as that of calcium carbonate and its utilization in either form is equally improved by D. The biochemical behavior of lead is influenced by dietary vitamin D as well as by calcium and phosphorus (138).

Since its effectiveness is variable, the requirements for D cannot be stated categorically. The need of dogs is about the same as that of chicks (139); for growing pigs, D is indispensable even with high intakes of calcium and phosphorus (140), and white pigs gain an advantage from daylight that black and brown ones do not (141). The requirements of turkeys have received further study (142), and typical low-calcium rickets has been produced in the guinea pig (143). Deficiencies of D are common in livestock (144) and are as serious as mineral deficiencies.

Cow milk contained an insignificant portion (1 to 2 per cent) of the vitamin D that was fed in the diet of hay (145). The administration of D to lactating Jersey and Holstein cows gave the same concentration of D in the blood and butterfat of both, but since the former produced more butterfat the yield of D was higher (146). The D in ewe milk was temporarily increased during the feeding of irradiated yeasts and molds; it was raised less by irradiated ergosterol (147). Human milk contained an average of 6 I.U. per 100 cc., influenced by diet but not by season (148). Irradiated evaporated milk is a valuable source of D in infant feeding (149), but alone may not always furnish optimum amounts. Irradiated milks given to children in good health and on good diets stimulate skeletal development (150). The maximum effect of vitamin D on the linear growth of infants was obtained with doses of 300 to 600 U.S.P. units, and this was paralleled by the response in dental eruption (151). As determined by the line test there was no seasonal variation in the D content of blood serum of children and adults (152); the normal range was from 66 to 165 U.S.P. units per 100 cc. of serum.

Comparisons of the relative effectiveness of D_2 and D_3 have given variable answers. As a prophylactic against rickets D_3 was much more efficient (153), but both were equally effective in healing rachitic babies and children (154), in curing osteomalacia and late rickets (155), and in the control of a case of congenital thyroid and para-

thyroid deficiency (156). It has been claimed that for the cure of rickets D_2 must be given by mouth, but D_3 may be given intramuscularly (157). Single massive doses of D_2 gave none but favorable results in the treatment of rickets, tetany, and spasmophilia (158). Excessive amounts of D_2 and D_3 were most harmful to rats (200 U.S.P. units per gm. daily) when the ratio of calcium to phosphorus in the diet was normal; they were somewhat toxic with a diet low in phosphorus but adequate in calcium, and they were favorable on diets low in calcium and adequate in phosphorus (159); lack of A accentuated the damage. Recovery was more rapid after administration of an excess of D_3 than of D_2. Chronic mild hyperparathyroidism in the absence of D appeared advantageous to animals on a diet low in phosphorus, producing an effect similar to that of cod-liver oil. Avitaminosis D decreased the thyrotropic hormone of the pituitary (160). Both pituitary and thyroid were found necessary for the calorigenic action of vitamin D which is independent of its effect on mineral metabolism (161).

Several miscellaneous observations included the effect of vitamin D_3 on hibernation (162) and the involvement of D, calcium, and phosphorus in the etiology of myopia (163). There was no correlation between phosphatase and the clinical picture in arthritis patients receiving relief through vitamin D therapy (164). The addition of D to various rat diets decreased the incidence of fissure caries, but since fats and fatty oils not containing D had similar effects, the action was considered local rather than systemic (165). The role of vitamin D in calcium metabolism in osteomalacia has been studied and reviewed (166). Also the relation of vitamins to dental caries has been summarized (167). The chemistry, physiology, pharmacology, and pathology of D have been presented in a comprehensive and informative book (168).

Vitamin E

Uncertainty as to the nature of γ-tocopherol has been dispelled by the demonstration (169) that it is 7,8-dimethyltocol. Various derivatives of α-tocopherol have been synthesized (170): 5,7-dimethyl-δ-ethyltocol; a diethyl-methyltocol; 3,4(?)-dehydro-(chromene) α-tocopherol; a phosphate ester of α-tocopherol (the sodium salt of which is water-soluble) possessing biological activity equal to α-tocopherol although it is only slowly hydrolyzable *in vitro;* and a compound lacking two isoprene units in the side chain which was in-

active in amounts of 40 mg. An improved procedure for the synthesis of α-tocopherol (171) gave a larger yield of more readily purifiable material. A new method of synthesis also was found in a reduction of 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone (172). The biologically inactive quinone obtained by oxidation with nitric acid or silver nitrate (Furter & Meyer) is an ortho- and not a para-quinone (173).

The apparent oxidation potential of α-tocopherol was measured by two methods (174) and was found to be between the normal oxidation potentials of mono- and dimethyl hydroquinone. Oxidation to tocopherylquinone seems to be irreversible in the organism, since tocopherylquinone cannot replace tocopherol in re-establishing fertility in E-deficient female rats, or in preventing nutritional muscular dystrophy in rabbits (174, 175). With wheat-germ oil or its unsaponifiable fraction as starting material, monotocopheryl succinates were prepared (176) the sodium salts of which are water-soluble.

When they can be further improved and simplified, chemical methods may presently displace biological methods of assay. The potentiometric method has been successfully applied to unsaponified wheat-germ oil (177), and figures obtained on various animal tissues and lard after saponification (178) were in fair agreement with those obtained by the colorimetric method (ferric chloride and α-α-dipyridyl) unless the solutions were highly colored. Assays by the colorimetric method were in good agreement with biological assays (179), but the possible destruction of tocopherol during saponification may not be overlooked (180). The colorimetric method has been applied to material containing very small amounts of tocopherol, such as blood serum (181). Spectroscopic methods are fraught with difficulties but after oxidation by silver nitrate, 0.01 per cent of tocopherol can thus be detected (182).

As has been known, the biological test suffers from the fact that the relation between dosage and response is not uniform, and some of the difficulties in determining the mean fertility dose have been described (183). The diminished effectiveness of a dose with increasing age of the animal has been indicated quantitatively (184); an increasing failure of implantation after successive resorptions has again been reported (185). The use of virgin rats is thus advantageous, although possible variations in the tocopherol stores must be avoided by controlled rearing, particularly to prevent access to vitamin E before weaning and transfer to the E-deficient diet. A recent extensive study (186) has shown that placental transmission in the rat is exceedingly

limited, whereas the vitamin is somewhat more readily transferred through the mammary gland. The possible use of *Daphnia magna* for rapid detection and evaluation of E (187) seems not to have been further developed.

Little progress has been made toward an understanding of the functions of vitamin E in the organism. The claim for thyroid subnormality in rats low in vitamin E was not substantiated (188). Pituitary changes which are characteristic in E-deficient male animals seem to appear even though fertility is retained by minimal doses (189). The effects of E-deficiency can thus far not be correlated with hormonal imbalance (190).

The importance of E for normal growth was again confirmed (191). The stated minimum daily requirement of α-tocopherol shows wide variation, from 0.075 to 0.75 mg. (192, 193). Even in the fourth (maternal) generation of animals on an E-deficient diet, fertility was maintained in male offspring through prophylactic administration of wheat-germ oil (194). There appears to be a sexual difference in the utilization or storage of vitamin E by young rats (186), and it is this difference (193) which has prompted the erroneous belief that there is a sex difference in minimum requirements. The stores of vitamin A in the liver are greater when the diet contains vitamin E than when it does not (195), and the continuous ingestion of mineral oil greatly reduces these stores and reproductive efficiency (196). The period of gestation is often prolonged in the presence of borderline amounts of vitamin E (197), and resorptions under those conditions may lead to toxemia due to the absorption of fetal material (198). The histology of uterine musculature of E-deficient rats is characterized by the deposition of a brownish pigment and subsequent degeneration, and the convoluted tubules of the kidney also show some degeneration (199). To insure satisfactory reproduction goats do not require rations supplemented with E (200). The female mouse responds to E deprivation as the rat does, but the male does not and shows no testicular damage after prolonged lack (201). The feeding of E-deficient diets to various species of domestic fowls is followed by curiously varied results, among which muscle pathology is often the least striking symptom and may be quite absent (202), but these diverse pathological conditions have thus far not provided any comprehensive outlook, unless this be an increase in capillary permeability (203).

The problem is complicated by the fact that other factors associated

with E-deficient diets may play a role. The commonly used E-deficient rations all contain lard or cod-liver oil, and the frequent practice of allowing them to stand for some days insures the disappearance of the last traces of E, probably by coupled fat oxidation. Various partial oxidation products of fats, among them aldehydes, have been credited with interrupting pregnancy, not by destruction of vitamin E, but by their direct effect upon the embryos (204). The administration of relatively small doses of heptaldehyde intraperitoneally to mice on an adequate stock diet produced resorptions even when the treatment was begun as late as the thirteenth day of gestation (205). If such toxic factors are associated with the manifestations of prolonged vitamin-E deprivation, the effects must be segregated by further experimentation.

The alleged carcinogenic action of wheat-germ oil has again been denied (206). The incidence of spontaneous mammary tumors in mice was less on E-deficient diets (207). Mammary carcinoma maintained in E-deficient rats for $2\frac{1}{2}$ years by 22 successive implantations showed no change in character (208). Jensen and Brown-Pierce sarcomas contain much more E than normal tissues do (209).

The relation of vitamin E to nutritional muscular dystrophy has received much attention and for the first time dystrophy has been produced in puppies (210). Further work on young rabbits (211, 212), guinea pigs (213), rats (214), and ducklings (215) has confirmed the prophylactic and curative effects of α-tocopherol. In young rats, occasional spontaneous recovery is accompanied by regeneration of musculature (216). The administration of E to old, E-deficient rats arrested the development of symptoms but was not curative (217).

Work on rabbits has been particularly extensive (212), confirming the altered metabolism of creatine but showing that the increased urinary output did not necessarily parallel the severity of the disease. The alleged multiple nature of the deficiency has been disproved (218); no water-soluble factors beyond those present in the basal diet were necessary for a cure; the minimum requirement of α-tocopherol in rabbits is uncertain (212, 219). The probable importance of E in muscle metabolism can be judged from the effects caused by its absence: the altered chemical composition; decreased contractile power (220); and increased oxygen uptake (221). Minor histological changes may exist without external symptoms becoming manifest (222, 223). The muscle damage produced in guinea pigs is believed to be secondary to anterior horn cell degeneration (224). The pre-

vention of dystrophic lesions in young rats by nerve section (225) is open to several interpretations.

The use of wheat germ in muscular and nervous diseases (226) was favorably reported on as was the use of α-tocopherol in amyotrophic lateral sclerosis and in malnutrition (227), but it is still doubtful whether any of the clinical lesions of muscle or nerve is typical of those produced experimentally by lack of vitamin E. The discussion of the relation of vitamin E to human reproduction has been continued (228), but the claims put forward were not acceptable to the Council on Pharmacy and Chemistry of the American Medical Association (229). Furthermore, the increased amount of antiproteolytic estrogenic factor in the serum of E-deficient rats as compared with normal animals, which originally served as a basis of differentiation (230), was not confirmed (231), nor did the addition of theelin to normal serum suppress tryptic proteolysis. As judged by animal tests, even massive doses of dl-α-tocopherol or its acetate are nontoxic (232).

Several reviews have appeared dealing with the chemistry (233) and physiology (222, 234) of vitamin E, the British Nutrition Panel Symposium (235) being especially comprehensive.

Vitamin K

The chemistry of vitamin-K compounds has been studied assiduously, especially the preparation of new derivatives having possible biological activity. Much of the information is primarily of interest to organic chemistry rather than to biochemistry.

Two of the three previously announced syntheses of K_1 have been described in greater detail (236). Under appropriate conditions sufficiently reactive β-unsaturated alcohols, dienes, or aryl carbinols were condensed with 2-alkyl-1,4-naphthohydroquinones, extensive cyclization to compounds of the tocopherol type being avoided (237); the substituted hydroquinones were isolated and converted into quinones of the K_1 type.

By means of other condensing agents further antihemorrhagic compounds have been prepared (238), among them 2-phytyl-1,4-naphthoquinone; 2-farnesyl- and 2-geranyl-compounds; and naphthotocopherol. All were less active biologically than K_1. A similar condensation of butadiene and toluquinone produced butadiene-toluquinone which had marked biological activity; this on reduction and

condensation with phytol gave 2-methyl-3-phytyl-5,8-dihydro-1,4-naphthohydroquinone and, by oxidation, the quinone, 5,8-dihydro derivative of vitamin K_1. This and the corresponding compound lacking the 3-phytyl group had antihemorrhagic action.

The same was true of a by-product of the K_1 synthesis which proved to be 2-methyl-2-phytyl-2,3-dihydro-1,4-naphthoquinone, isomeric with naphthotocopherol (239); it was converted in small part to K_1 by pyrolysis. The 2- and 3-methyl-7-naphthols were found to have striking biological activity whereas the 1- and 3-methyl-2-naphthols did not (240). Simple and convenient procedures for the preparation of antihemorrhagic compounds have been described (241).

By quite a different method 2-phytyl-1,4-naphthoquinone (*nor*-vitamin K_1, *nor*-α-phylloquinone) was synthesized and another and general method for the preparation of 2-methyl-3-alkyl naphthoquinones was outlined in detail (242). Among the new quinones thus synthesized 2-methyl-3-octadecyl-, and 2-methyl-3-dihydrophytyl-, derivatives of 1,4-naphthoquinone were slightly active.

Hydrogen peroxide was found to be a suitable oxidizing agent for the preparation of naphthoquinone oxides, of which several were found to be very effective biologically (243); reduction to the corresponding quinones, hydroquinones, and hydrides, was easily accomplished. The oxidation-reduction potential of K_1 determined indirectly with corrections was 363 mv. at 20° (244).

On the basis of degradation products a structural formula was proposed for vitamin K_2 ($C_{41}H_{56}O_2$) (245); this structure differs from that of K_1 only in having a difarnesyl side chain in place of the phytyl side chain of K_1. A brief note on the synthesis of K_2 as a 2,3-difarnesyl derivative (246) has not been further elaborated.

Many water-soluble forms of vitamin K, when compared on a molecular basis, were as active as 2-methyl-1,4-naphthoquinone (247) or more so, when given intravenously or subcutaneously. Among those tested were: the 4-amino-2- or 3-methyl-1-naphthols (as hydrochlorides) (248); the addition product of 2-methyl-1,4-naphthoquinone and sodium bisulfite; 1,4-dihydroxy-2-methyl-3-naphthaldehyde (249); various types of esters, including the stable salts of disulfuric and diphosphoric esters of reduced K_1 and of 2-methyl-1,4-naphthohydroquinone (250); 2-methyl-1,4-disuccinyl-naphthohydroquinone (251, 252); and 2-methyl-1,4-naphthylenedioxydiacetic acid (247). Successful results were obtained both in animals and man by administration *per os* without the use of bile salts (253). Injections of

emulsified K_1 were equally effective in chicks, whether the hypoprothrombinemia had been produced by diet or by bile duct ligation (254). Toxic dosages of some of the synthetic K compounds are far above the amounts needed therapeutically (255).

To secure absorption of K from the alimentary tract, agencies other than bile salts have been sought. In bile-fistula rats the choleic acid of K was only partially successful (256); with patients, dioctyl sodium sulfosuccinate in capsules with 2-methyl-1,4-naphthoquinone was effective whereas the K compound alone was not (257). High intakes of mineral oil tended to produce prothrombin deficiency in rats, corrected by subcutaneous administration of K (258). The supposedly desirable addition of adsorbing charcoals to chick diets may give rise to deficiencies of various kinds, among them of K, depending on the type of carbon used (259).

No progress has been made in the development of a chemical method of assay and perhaps none may be expected until the molecular configurations responsible for K activity are better understood. The biochemical assays by different investigators have continued to lack agreement, but with variations in the manner of conducting the assay, six or eighteen hours (260), and with possible variation in rates of absorption, utilization, and excretion of different compounds, it is surprising to find as close agreement as has recently been reported (261). Another series of comparative bio-assays has appeared (262) together with color plates of the pathological manifestations of deficiency and description of parenchymal damage to liver and kidney. A large number of quinone derivatives, including some not heretofore mentioned, have been carefully assayed (251) and the results discussed. The various preventive and curative techniques have been compared with a view to converting the various units, one into another (263).

Although K activity may not be confined to 2-methyl-1,4-naphthoquinones and substances which can be converted to this in the organism (264), those which lack the 2-methyl group have relatively low biological activity (251). The highest potency so far obtained is generally agreed to reside in 2-methyl-1,4-naphthoquinone (265), and the proposed general adoption of this substance, or of a more stable form, such as an ester of the hydroquinone (251), as a universal standard for biological assays will be a distinct advance.

A simplified modification of Howell's prothrombin time, a one-stage determination, has been proposed to determine vitamin-K de-

ficiency (266). A new type of experimental K-deficient diet containing a heated grain mixture has also been described (267).

Vitamin-K compounds, notably K_2, are known to be produced by bacteria and appear in the rumen of cows on K-deficient diets (268). K is indispensable for the growth of Johne's bacillus (*Mycobacterium paratuberculosis*) (269). It is not necessary for the growth of yeast. It is found in the chloroplasts and not in the cytoplasm of cabbage cells (270).

Many clinical observations, too many to cite, have continued to demonstrate that hypoprothombinemia, whether associated with obstructive jaundice or other unfavorable alimentary or dietary conditions, yields to the administration of K in various forms and by various pathways. The failure of patients to respond is accepted as presumptive evidence of severe liver damage since such failure is encountered following experimental liver damage produced in dogs and cats by chloroform or carbon tetrachloride (271). The importance of the liver as the place of origin of prothrombin and perhaps of storage of K is thus emphasized (272).

The well-recognized deficiency in plasma prothrombin of newborns is particularly marked in premature infants (273). There are many reports, again too numerous to mention, of the successful use of various K compounds given to mothers during labor or to infants directly after birth; the compounds differed in their effectiveness, depending also on the method of administration. The importance of such prophylaxis deserves wide-spread attention.

Comprehensive and useful reviews have appeared on blood coagulation and vitamin K (274).

LITERATURE CITED

VITAMIN A

1. GRAY, E. L., AND CAWLEY, J. D., *J. Biol. Chem.*, **134**, 397 (1940)
2. EMBREE, N. D., AND SHANTZ, E. M., *J. Biol. Chem.*, **132**, 619 (1940)
3. CARTER, G. P., AND GILLAM, A. E., *Biochem. J.*, **33**, 1325 (1939)
4. KARRER, P., AND SCHWAB, G., *Helv. Chim. Acta*, **23**, 578 (1940)
5. KARRER, P., AND RÜEGGER, A., *Helv. Chim. Acta*, **23**, 284 (1940)
6. KARRER, P., *Helv. Chim. Acta*, **22**, 1149 (1939)
6a. MASON, K. E., in *Sex and Internal Secretions* (ALLEN), 2nd ed., Chap. XXII (1939)
7. BAXTER, J. G., AND ROBESON, C. D., *Science*, **92**, 203 (1940)
7a. FIXSEN, M. A. B., AND ROSCOE, M. H., *Nutrition Abstracts & Revs.*, **9**, 795 (1940)

8. TAUBER, H., *J. Am. Chem. Soc.*, **62**, 2251 (1940); SUMNER, J. B., AND SUMNER, R. J., *J. Biol. Chem.*, **134**, 531 (1940)

9. LEASE, E. J., LEASE, J. G., WEBER, J., AND STEENBOCK, H., *J. Nutrition*, **16**, 571 (1938); SIMONS, E. J., BUXTON, L. O., AND COLMAN, H. B., *Ind. Eng. Chem., Ind. Ed.*, **32**, 706 (1940); HARRELSON, R. T., NELSON, P. M., LOWE, B., DYME, H. C., AND NELSON, V. E., *Iowa State Coll. J. Sci.*, **13**, 353 (1939)

10. PETERING, H. G., MORGAL, P. W., AND MILLER, E. J., *Ind. Eng. Chem., Ind. Ed.*, **32**, 1407 (1940)

11. WITH, T. K., *Nord. Med.*, **3**, 2903 (1939)

12. GORTER, F. J., AND SPRUYT, J. P., *Arch. néerland. physiol.*, **24**, 122 (1939)

13. BACHARACH, A. L., *Biochem. J.*, **34**, 542 (1940)

14. LUNDE, G., AND KRINGSTAD, H., *Tids. Kjemi Bergvesen*, **20**, 14 (1940)

15. BARTHE, C. L., BERG, F. F., CARTER, E. B., COPLEY, D. M., FOSBINDER, R. J., LEWIS, T., AND TAYLOR, F. O., *J. Am. Pharm. Assoc.*, **28**, 661 (1939)

16. WILKIE, A. J. B., *J. Assoc. Official Agr. Chem.*, **22**, 465 (1939); **23**, 336 (1940)

17. FRENCH, R. B., *Ind. Eng. Chem., Anal. Ed.*, **12**, 351 (1940)

18. NEISH, A. C., McFARLANE, W. D., AND BRECHIN, W. A., *Can. J. Research, B*, **17**, 325 (1939); PETERING, H. G., WOLMAN, W., AND HIBBARD, R. P., *Ind. Eng. Chem., Anal. Ed.*, **12**, 148 (1940); MOORE, L. A., *Ind. Eng. Chem., Anal. Ed.*, **12**, 726 (1940)

19. FRAPS, G. S., KEMMERER, A. R., AND GREENBERG, S. M., *Ind. Eng. Chem., Anal. Ed.*, **12**, 16 (1940); *J. Assoc. Official Agr. Chem.*, **23**, 422, 659 (1940)

20. LEASE, E. J., AND MITCHELL, J. H., *Ind. Eng. Chem., Anal. Ed.*, **12**, 337 (1940)

21. KOEHN, C. J., AND SHERMAN, W. C., *J. Biol. Chem.*, **132**, 527 (1940)

22. DANN, W. J., AND EVELYN, K. A., *Biochem. J.*, **32**, 1008 (1938)

23. EDISBURY, J. R., *Analyst*, **65**, 484 (1940); EWING, D. T., VANDENBELT, J. M., EMMETT, A. D., AND BIRD, O. D., *Ind. Eng. Chem., Anal. Ed.*, **12**, 639 (1940); McFARLAN, R. L., BATES, P. K., AND MERRILL, E. C., *Ind. Eng. Chem., Anal. Ed.*, **12**, 645 (1940)

24. WESTENBERG, L., *Verslag. Landb. Onderzoek., E*, No. 45, 587 (1939)

25. MOORE, L. A., *J. Dairy Sci.*, **22**, 501 (1939); LANZING, J. C., *Arch. néerland. physiol.*, **24**, 112 (1939); VANVEEN, A. G., AND LANZING, J. C., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **10**, 6 (1940)

26. JOSEPHS, H. W., *Bull. Johns Hopkins Hosp.*, **65**, 112 (1939)

27. SEABER, W. M., *Analyst*, **65**, 266 (1940); MORTON, R. A., *Chemistry & Industry*, 301 (1940)

28. PETERSON, W. J., HUGHES, J. S., AND PAYNE, L. F., *Kansas Agr. Expt. Sta., Tech. Bull.*, **46**, 3 (1939)

29. KUHN, R., STENE, J., AND SORENSEN, N. A., *Ber.*, **72B**, 1688 (1939)

30. JENSEN, H. B., AND WITH, T. K., *Biochem. J.*, **33**, 1771 (1939)

31. TISCHER, J., *Z. physiol. Chem.*, **260**, 257 (1939); SCHEUNERT, A., AND WAGNER, K. H., *Z. physiol. Chem.*, **260**, 272 (1939)

32. RANDOLPH, L. F., AND HAND, D. B., *J. Agr. Research*, **60**, 51 (1940)

33. CASTELLANOS, A. O., *Anales asoc. quim. argentina*, **28**, 91 (1940); HERMS-

DORFF, G., AND PECKOLT, O. DE L., *Bol. ministerio agr. (Brazil)*, **28**, 83 (1939); CUNNINGHAM, M. M., AND SLATER, E. C., *Australian J. Exptl. Biol. Med. Sci.*, **17**, 457 (1939); SESHAN, P. K., *Indian J. Med. Research*, **27**, 711 (1940); BASU, K. P., SIRCAR, B. C. R., AND GUPTA, J. C. S., *Indian J. Med. Research*, **27**, 721 (1940)

34. PUGSLEY, L. I., *J. Fisheries Research Board Can.*, **4**, 396 (1939); IOVEN-SKIOLD, H., *Tids. Kjemi Bergvesen*, **19**, 118 (1939); RUSOFF, L. L., AND MEHRHOF, N. R., *Poultry Sci.*, **18**, 339 (1939); MOLTENO, C. J., AND RAPSON, W. S., *Biochem. J.*, **33**, 1390 (1939)

35. SJOLLEMA, B., AND DONATH, W. F., *Biochem. J.*, **34**, 736 (1940)

36. SUOMALAINEN, P., *Suomen Kemistilehti, B*, **12**, 30 (1939)

37. CLEGG, R. E., AND SATTERFIELD, G. H., *J. Elisha Mitchell Sci. Soc.*, **55**, 241 (1939); HENRY, K. M., HOUSTON, J., AND KON, S. K., *J. Dairy Research*, **11**, 1 (1940)

38. MAITRA, M. K., AND MOORE, T., *Biochem. J.*, **33**, 1648 (1939)

39. MACKAY, H. M. M., *Arch. Disease Childhood*, **14**, 245 (1939)

40. WITH, T. K., *Nord. Med.*, **3**, 2468 (1939); SMITH, M. C., AND SPECTOR, H., *Ariz. Agr. Expt. Sta., Tech. Bull.*, **84**, 373 (1940)

41. ANDERSEN, O., *Klin. Wochschr.*, **18**, 499 (1939)

42. CURTIS, A. C., AND BALLMER, R. S., *J. Am. Med. Assoc.*, **113**, 1785 (1939); CURTIS, A. C., AND HORTON, P. B., *Am. J. Med. Sci.*, **200**, 102 (1940)

43. GRAY, E. L., MORGAREIDGE, K., AND CAWLEY, J. D., *J. Nutrition*, **20**, 67 (1940)

44. GRAY, E. L., HICKMAN, K. C. D., AND BROWN, E. F., *J. Nutrition*, **19**, 39 (1940)

45. WITH, T. K., *Nord. Med.*, **3**, 2901 (1939)

46. GROTH, H., AND SKURNIK, L., *Acta Med. Scand.*, **101**, 333 (1939)

47. MAJUMDAR, B. N., *Indian J. Med. Research*, **27**, 413 (1939)

48. WITH, T. K., *Nord. Med.*, **3**, 2906 (1939)

49. SUOMALAINEN, P., *Skand. Arch. Physiol.*, **83**, 104 (1939)

50. SUOMALAINEN, P., *Skand. Arch. Phyiol.*, **83**, 94 (1939); KLAR, E., *Z. ges. exptl. Med.*, **105**, 161 (1939)

51. KREULA, M., AND VIRTANEN, A. I., *Upsala Läkarefören. Förh.*, **45**, 355 (1939)

52. BOOHER, L. E., AND CALLISON, E. C., *J. Nutrition*, **18**, 459 (1939)

53. GUILBERT, H. R., HOWELL, C. E., AND HART, G. H., *J. Nutrition*, **19**, 91 (1940)

54. HART, G. H., *Nutrition Abstracts & Revs.*, **10**, 261 (1940)

55. MOORE, L. A., *J. Dairy Sci.*, **22**, 803 (1939)

56. WARD, R. E., BECHDEL, S. I., AND GUERRANT, N. B., *J. Dairy Sci.*, **23**, 115 (1940); CONVERSE, H. T., AND MEIGS, E. B., *Proc. Am. Soc. Animal Production*, **32**, 67 (1939)

57. RIGGS, J. K., *J. Nutrition*, **20**, 491 (1940); DAS-GUPTA, S. M., *Indian J. Vet. Sci.*, **9**, 249 (1939); WILBUR, J. W., HILTON, J. H., AND HAUGE, S. M., *J. Dairy Sci.*, **23**, 765 (1940); JOHNSON, B. C., AND PETERSON, W. H., *J. Dairy Sci.*, **23**, 385 (1940)

58. HAUGE, S. M., HILTON, J. H., AND WILBUR, J. W., *J. Dairy Sci.*, **23**, 719 (1940)

59. WITH, T. K., AND WANSCHER, O., *Nord. Med.*, **4**, 3425 (1939); WIL-

LIAMS, J. K., LAMPMAN, C. E., AND BOLIN, D. W., *Poultry Sci.*, **18**, 268 (1939); SHERWOOD, R. M., AND FRAPS, G. S., *Texas Agr. Expt. Sta. Bull.*, **583**, 5 (1940)

60. TEPPER, A. E., AND DURGIN, R. C., *New Hampshire Agr. Expt. Sta. Bull.*, **310**, 3 (1938)
61. VERMES, M., LISOT, G., MEUNIER, P., AND RAOUL, Y., *Compt. rend. acad. agr. France*, **25**, 1090 (1939); VERMES, M., MEUNIER, P., AND RAOUL, Y., *Compt. rend.*, **209**, 578 (1939)
62. LINTON, R. G., AND BROWNLEE, A., *Nature*, **144**, 978 (1939)
63. RUSSELL, W. C., AND MORRIS, M. L., *J. Am. Vet. Med. Assoc.*, **95**, 316 (1939)
64. COOMBES, A. I., OTT, G. L., AND WISNICKY, W., *North Am. Veterinarian*, **21**, 601 (1940)
65. GOSS, H., AND GUILBERT, H. R., *J. Nutrition*, **18**, 169 (1939)
66. GRIDGEMAN, N. T., LEES, H., AND WILKINSON, H., *J. Soc. Chem. Ind.*, **59**, 120 (1940)
67. LUCE-CLAUSEN, E. M., AND BROWN, E. F., *J. Nutrition*, **18**, 537 (1939)
68. KAO H. C., AND SHERMAN, H. C., *Proc. Soc. Exptl. Biol. Med.*, **45**, 589 (1940)
69. KRAKOWER, C., AND AXTMAYER, J. H., *Proc. Soc. Exptl. Biol. Med.*, **45**, 583 (1940)
70. GOERNER, A., AND GOERNER, M. M., *J. Nutrition*, **18**, 441 (1939)
71. BOWERS, R. E., AND McCAY, C. M., *Science*, **92**, 291 (1940)
72. LUND A., *180de Berein. Forsögslab.*, 1 (1938)
73. MOORE, L. A., AND SYKES, J. F., *Am. J. Physiol.*, **130**, 684 (1940)
74. FRIDERICIA, L. S., GUDJONSSON, S., VIMTRUP, B., CLEMMESEN, S., AND CLEMMESEN, J., *Am. J. Cancer*, **39**, 61 (1940)
75. IRVING, J. T., AND RICHARDS, M. B., *Biochem. J.*, **34**, 198 (1940)
76. KING, J. D., *Brit. Dental J.*, **68**, 349 (1940); MELLANBY, H., *Brit. Dental J.*, **67**, 187 (1939); IRVING, J. T., AND RICHARDS, M. B., *Nature*, **144**, 908 (1939)
77. MUTCH, J. R., AND RICHARDS, M. B., *Brit. J. Ophthalmol.*, **23**, 381 (1939)
78. ROKHLINA, M. L., *Bull. biol. méd. exptl. U.R.S.S.*, **3**, 362 (1937); SMITH, D. C., AND PERMAN, J. M., *Endocrinology*, **27**, 110 (1940); WESLAW, W., AND WROBLEWSKI, B., *Z. ges. exptl. Med.*, **105**, 497 (1939); SCHULZE, E., AND HUNDHAUSEN, G., *Arch. exptl. Path. Pharmakol.*, **192**, 43 (1939); BELASCO, I. J., AND MURLIN. J. R., *J. Nutrition*, **20**, 577 (1940); BAUMANN, C. A., AND MOORE, T., *Biochem. J.*, **33**, 1639 (1939)
79. SUTTON, T. S., AND BRIEF, B. J., *Endocrinology*, **25**, 302 (1939)
80. HUME, E. M., BURBANK, R., AND KORENCHEVSKY, V., *J. Path. Bact.*, **49**, 291 (1939)
81. HECHT, S., AND MANDELBAUM, J., *Am. J. Physiol.*, **130**, 651 (1940)
82. WALD, G., AND STEVEN, D., *Proc. Natl. Acad. Sci. U.S.*, **25**, 344 (1939)
83. JEANS, P. C., BLANCHARD, E. L., AND SATTERTHWAITE, F. E., *J. Pediat.*, **18**, 170 (1941)
84. ECKHARDT, R., AND JOHNSON, L. V., *J. Pediat.*, **18**, 195 (1941); HUNT, E. P., AND PALMER, C. E., *Milbank Mem. Fund Quart.*, **18**, 403 (1940)
85. WAGNER. K. H., *Z. physiol. Chem.*, **264**, 153 (1940)

86. BLANCHARD, E. L., AND HARPER, H. A., *Arch. Intern. Med.,* **66,** 661 (1940)

87. PETT, L. B., *J. Lab. Clin. Med.,* **25,** 149 (1939)

88. PETT, L. B., AND LEPAGE, G. A., *J. Biol. Chem.,* **132,** 585 (1940)

89. CLAUSEN, S. W., BAUM, W. S., McCOORD, A. B., RYDEEN, J. O., AND BREESE, B. B., *Science,* **91,** 318 (1940)

90. YOUNG, G., AND WALD, G., *Am. J. Physiol.,* **131,** 210 (1940)

91. PETT, L. B., *Science,* **92,** 63 (1940)

92. STEININGER, G., ROBERTS, L. J., AND BRENNER, S., *J. Am. Med. Assoc.,* **113,** 2381 (1939) ; KORB, J. H., *U.S. Naval Med. Bull.,* **37,** 392 (1939) ; FRONIMOPOULOS, J., *Klin. Monatsbl. Augenheilk.,* **104,** 1 (1940)

93. DRIGALSKI, W. V., *Z. Vitaminforsch.,* **9,** 325 (1939)

94. MACK, P. B., AND SANDERS, A. P., *Am. J. Med. Sci.,* **199,** 686 (1940)

95. CAUSSADE, L., NEIMANN, T., AND DAVIDSOHN, *Rev. franc. pediat.,* **14,** 209 (1938) ; LEHMAN, E., AND RAPAPORT, H. G., *J. Am. Med. Assoc.,* **114,** 386 (1940)

96. STEELE, E. J. P., *Lancet,* **II,** 205 (1940)

97. HIRST, J. C., AND SHOEMAKER, R. E., *Am. J. Obstet. Gynecol.,* **40,** 12 (1940)

98. BAER, H. L., AND VOGEL, H. R., *Urol. Cutaneous Rev.,* **44,** 176 (1940)

99. WOHL, M. G., AND FELDMAN, J. B., *J. Lab. Clin. Med.,* **25,** 485 (1940) ; THIELE, W., AND SCHERFF, I., *Klin. Wochschr.,* **18,** 1275 (1939) ; KHENTOV, YA. S., RACHEVSKII, F. A., AND SHENDEL, A. M., *Klin. Med. (U.S.S.R.),* **17,** 20 (1939)

100. PATEK, JR., A. J., AND HAIG, C., *J. Clin. Investigation,* **18,** 609 (1939)

101. BREESE, B. B., AND McCOORD, A. B., *J. Pediat.,* **16,** 139 (1940) ; SALAH, M., *J. Egypt. Med. Assoc.,* **23,** 153 (1940)

102. BRAZER, J. G., AND CURTIS, A. C., *Arch. Internal Med.,* **65,** 90 (1940)

103. RUDDY, M. V., *Studies Inst. Divi Thomae,* **2,** 165 (1939)

104. McDONALD, R., AND ADLER, F. H., *Arch. Ophthalmol. (Chicago),* **22,** 980 (1939)

105. HARRIS, L. J., AND ABBASY, M. A., *Lancet,* **II,** 1299 (1939) ; WILLIAMS, P. F., HARK, B., AND FRALIN, F. G., *Am. J. Obstet. Gynecol.,* **40,** 1 (1940) ; ISAACS, B. L., JUNG, F. T., AND IVY, A. C., *Arch. Ophthalmol. (Chicago),* **24,** 698 (1940)

106. HERRIN, R. C., AND NICHOLES, H. J., *J. Clin. Investigation,* **19,** 489 (1940)

107. DEROTTH, A., *Arch. Ophthalmol. (Chicago),* **24,** 281 (1940)

108. MILLER, H., *Am. J. Opthalmol.,* **23,** 296 (1940)

109. KON, S. K., AND MADDOCK, C., *Vet. Record,* **50,** 1693 (1938) ; UDD-STRÖMER, M., *Acta. Med. Scand.,* **102,** 214 (1939)

110. CRIMM, P. D., AND SHORT, D. M., *Ann. Internal Med.,* **13,** 61 (1939)

111. DARZINS, E., *Ann. inst. Pasteur,* **63,** 455 (1939)

112. YUDKIN, A. M., *Bull. N. Y. Acad. Med.,* **15,** 406 (1939)

VITAMIN D

113. WINDAUS, A., AND ROOSEN-RUNGE, C., *Z. physiol. Chem.,* **260,** 181 (1939) ; WINDAUS, A., DIMROTH, K., AND BREYWISCH, W., *Ann.,* **543,** 240 (1940)

114. BUNKER, J. W. M., HARRIS, R. S., AND MOSHER, L. M., *J. Am. Chem Soc.*, **62**, 508 (1940)

115. NAKAMIYA, Z., AND TAKIZAWA, K., *Bull. Inst. Phys. Chem. Research (Tokyo)*, **18**, 472 (1939)

116. CREWS, S. K., AND SMITH, E. L., *Analyst*, **64**, 568 (1939)

116a. TORTELLI, M., AND JAFFE, E., *Ann. chim. applicata*, **2**, 80 (1914)

117. SOLYANIKOVA, V. L., *Biokhimiya*, **4**, 483 (1939)

117a. BROCKMANN, H., AND CHEN, Y. H., *Z. physiol. Chem.*, **241**, 129 (1936)

118. RITSERT, K., *E. Merck's Jahresber.*, **52**, 27 (1938)

119. NIELD, C. H., RUSSELL, W. C., AND ZIMMERLI, A., *J. Biol. Chem.*, **136**, 73 (1940)

120. GRIDGEMAN, N. T., LEES, H., AND WILKINSON, H., *Analyst*, **65**, 493 (1940)

121. GRIEM, W. B., *J. Assoc. Official Agr. Chem.*, **22**, 656 (1939) ; SHRIMPTON, E. A. G., *Quart. J. Pharm. Pharmacol.*, **13**, 97 (1940) ; FRAPS, G. S., KEMMERER, A. R., MEINKE, W. W., AND GREENBERG, S. M., *J. Assoc. Official Agr. Chem.*, **23**, 417 (1940)

122. WESTERLUND, A., *Upsala Läkarefören. Förh.*, **45**, 33 (1939)

123. OLSSON, N., *Kgl. Fysiograf. Sällskap. Lund. Handl.*, **9**, 3 (1939) ; BAKER, A. Z., AND WRIGHT, M. D., *Analyst*, **65**, 326 (1940)

124. McCHESNEY, E. W., AND HOMBURGER, E., *J. Nutrition*, **20**, 339 (1940)

125. CARVER, J. S., HEIMAN, V., AND COOK, J. W., *Poultry Sci.*, **18**, 288 (1939)

126. RUSSELL, W. C., TAYLOR, M. W., AND DUNCAN, M. T., *J. Nutrition*, **18**, 27 (1939)

127. O'BRIEN, B., AND MORGAREIDGE, K., *J. Nutrition*, **18**, 277 (1939)

128. RUSSELL, W. C., *J. Assoc. Official Agr. Chem.*, **23**, 341 (1940)

129. COWARD, K. H., AND KASSNER, E. W., *Biochem. J.*, **34**, 538 (1940)

130. ADOLPH, W. H., *Chinese J. Physiol.*, **14**, 51 (1939)

131. KNUDSON, A., AND FLOODY, R. J., *J. Nutrition*, **20**, 317 (1940)

132. MORGAREIDGE, K., AND FINN, S. B., *J. Nutrition*, **20**, 75 (1940)

133. MORGAREIDGE, K., AND MANLY, M. L., *J. Nutrition*, **18**, 411 (1939)

134. TWEEDY, W. R., TEMPLETON, R. D., PATRAS, M. C., AND McJUNKIN, F. A., *Endocrinology*, **26**, 481 (1940)

135. SMITH, M. C., AND SPECTOR, H., *J. Nutrition*, **20**, 19, 197 (1940)

136. JONES, J. H., *J. Nutrition*, **18**, 507 (1939)

137. KRIEGER, C. H., BUNKFELDT, R., AND STEENBOCK, H., *J. Nutrition*, **20**, 7, 15 (1940) ; KRIEGER, C. H., AND STEENBOCK, H., *J. Nutrition*, **20**, 125 (1940)

138. SOBEL, A. E., YUSKA, H., PETERS, D. D., AND KRAMER, B., *J. Biol. Chem.*, **132**, 239 (1940)

139. ARNOLD, A., AND ELVEHJEM, C. A., *J. Am. Vet. Med. Assoc.*, **95**, 187 (1939)

140. SENIOR, B. J., *Sci. Proc. Roy. Dublin Soc.*, **22**, 229 (1940)

141. JOHNSON, D. W., AND PALMER, L. S., *J. Agr. Research*, **58**, 929 (1939)

142. JUKES, T. H., AND SANFORD, T. D., *J. Nutrition*, **18**, 71 (1939)

143. HOWE, P. R., WESSON, L. G., BOYLE, P. E., AND WOLBACH, S. B., *Proc. Soc. Exptl. Biol. Med.*, **45**, 298 (1940)

144. HAWTHORNE, G. A., *North Am. Veterinarian*, **21**, 342 (1940)

145. WALLIS, G. C., *J. Dairy Sci.*, **22**, 599 (1939)

146. WALLIS, G. C., *Proc. S. Dakota Acad. Sci.,* **19**, 55 (1939)

147. KIRK, W. G., *Iowa State Coll. J. Sci.,* **13**, 235 (1939)

148. DRUMMOND, J. C., GRAY, C. H., AND RICHARDSON, N. E. G., *Brit. Med. J.,* **II**, 757 (1939)

149. ELLIS, R. W. B., *Arch. Disease Childhood,* **14**, 295 (1939)

150. HUMMEL, F. C., HUNSCHER, H. A., AND MACY, I. G., *Am. J. Diseases Children,* **58**, 753 (1939)

151. JEANS, P. C., AND STEARNS, G., *J. Pediat.,* **13**, 730 (1938); SPEIDEL, T. D., AND STEARNS, G., *J. Pediat.,* **17**, 506 (1940)

152. WARKANY, J., AND MABON, H. E., *Am. J. Diseases Children,* **60**, 606 (1940)

153. HARTENSTEIN, H. J., *Deut. med. Wochschr.,* **66**, 143 (1940)

154. MORRIS, S., AND STEVENSON, M., *Lancet,* **II**, 876 (1939)

155. WILSON, D. C., *Lancet,* **I**, 961 (1940)

156. HIMSWORTH, H. P., AND MAIZELS, M., *Lancet,* **I**, 959 (1940)

157. NITSCHKE, A., *Z. Kinderheilk,* **61**, 385 (1939)

158. VOLLMER, H., *J. Pediat.,* **14**, 491 (1939); GUNNARSON, S., *Acta Paediat.,* **25**, 69 (1939); STRÖM, J., *Acta Paediat.,* **25**, 251 (1939)

159. MORGAN, A. F., SHIMOTORI, N., AND HENDRICKS, J. B., *J. Biol. Chem.,* **134**, 761 (1940)

160. SCHULZE, E., AND FLACH, M., *Arch. exptl. Path. Pharmakol.,* **193**, 727 (1939)

161. BARTOLI, A., FELDMAN, J., AND REED, C. I., *Am. J. Physiol.,* **127**, 552 (1939)

162. CASSENS, A., *Z. ges. exptl. Med.,* **106**, 521 (1939)

163. KNAPP, A., *Am. J. Ophthalmol.,* **22**, 1329 (1939)

164. SMITH, P. W., KLEIN, A. D., AND STECK, I. E., *J. Lab. Clin. Med.,* **24**, 1227 (1939)

165. ROSEBURY, E., AND KARSHAN, M., *J. Dental Research,* **18**, 189 (1939)

166. MAXWELL, J. P., PI, H. T., LIU, H. A. C., AND KUO, C. C., *Proc. Roy. Soc. Med.,* **32**, 287 (1939); CHU, H. I., LIU, S. H., YU, T. F., HSU, H. C., CHENG, T. Y., AND CHAO, H. C., *J. Clin. Investigation,* **19**, 327, 349 (1940); LIU, S. H., *Chinese Med. J.,* **57**, 101 (1940)

167. MELLANBY, M., AND KING, J. D., *Ergeb. Vitamin u. Hormonforsch.,* **2**, 1 (1939)

168. REED, C. I., STRUCK, H. C., AND STECK, I. E., *Vitamin D. Chemistry; Physiology; Pharmacology; Pathology; Experimental and Clinical Investigations* (University of Chicago Press, 1939)

VITAMIN E

169. EMERSON, O. H., AND SMITH, L. I., *J. Am. Chem. Soc.,* **62**, 1869 (1940)

170. KARRER, P., AND HOFFMANN, O., *Helv. Chim. Acta,* **22**, 654 (1939); **23**, 1126 (1940); KARRER, P., LEGLER, R. G., AND SCHWAB, G., *Helv. Chim. Acta,* **23**, 1132 (1940); KARRER, P., AND BUSSMANN, G., *Helv. Chim. Acta,* **23**, 1137 (1940); KARRER, P., AND YAP, K. S., *Helv. Chim. Acta,* **23**, 581 (1940)

171. SMITH, L. I., UNGNADE, H. E., STEVENS, J. R., AND CHRISTMAN, C. C., *J. Am. Chem. Soc.,* **61**, 2615 (1939)

172. FIESER, L. F., TISHLER, M., AND WENDLER, N. L., *J. Am. Chem. Soc.*, **62**, 2861 (1940)

173. SMITH, L. I., IRWIN, W. B., AND UNGNADE, H. E., *J. Am. Chem. Soc.*, **61**, 2424 (1939)

174. GOLUMBIC, C., AND MATTILL, H. A., *J. Biol. Chem.*, **134**, 535 (1940)

175. KARRER, P., AND GEIGER, A., *Helv. Chim. Acta*, **23**, 455 (1940); WRIGHT, M. D., AND DRUMMOND, J. C., *Biochem. J.*, **34**, 32 (1940); GOLUMBIC, C., AND MATTILL, H. A., *J. Biol. Chem.*, **135**, 339 (1940)

176. MCARTHUR, C. S., AND WATSON, E. M., *Can. Chem. Process Inds.*, **23**, 350 (1939)

177. KARRER, P., AND KELLER, H., *Helv. Chim. Acta*, **22**, 617 (1939)

178. KARRER, P., JAEGER, W., AND KELLER, H., *Helv. Chim. Acta*, **23**, 464 (1940)

179. ENGLE, C., AND EMMERIE, A., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **9**, 173 (1939)

180. EMMERIE, A., AND ENGEL, C., *Rec. trav. chim.*, **58**, 895 (1939)

181. EMMERIE, A., *Rec. trav. chim.*, **59**, 246 (1940)

182. MOORE, T., AND RAJAGOPAL, K. R., *Biochem. J.*, **34**, 335 (1940); CUTHBERTSON, W. F. J., RIDGEWAY, R. R., AND DRUMMOND, J. C., *Biochem. J.*, **34**, 34 (1940)

183. BACHARACH, A. L., *Biochem. J.*, **32**, 2017 (1938)

184. EMERSON, G. A., AND EVANS, H. M., *J. Nutrition*, **18**, 501 (1939)

185. WEBER, J., IRWIN, M. H., AND STEENBOCK, H., *Am. J. Physiol.*, **125**, 593 (1939)

186. MASON, K. E., AND BRYAN, W. L., *J. Nutrition*, **20**, 501 (1940)

187. VIEHOEVER, A., AND COHEN, I., *Am. J. Pharm.*, **110**, 297 (1938)

188. TELFORD, I. R., EMERSON, G. A., AND EVANS, H. M., *Proc. Soc. Exptl. Biol. Med.*, **38**, 623 (1938)

189. KONEFF, A., *Anat. Record*, **74**, 383 (1939)

190. DRUMMOND, J. C., NOBLE, R. L., AND WRIGHT, M. D., *J. Endocrinol.*, **1**, 275 (1939)

191. NELSON, M. M., EMERSON, G. A., AND EVANS, H. M., *Proc. Soc. Exptl. Biol. Med.*, **45**, 157 (1940)

192. EVANS, H. M., EMERSON, G. A., AND EMERSON, O. H., *Anat. Record*, **74**, 257 (1939)

193. MASON, K. E., *Am. J. Physiol.*, **131**, 268 (1940)

194. EMERSON, G. A., AND EVANS, H. M., *Proc. Soc. Exptl. Biol. Med.*, **45**, 159 (1940)

195. BACHARACH, A. L., *Quart. J. Pharm. Pharmacol.*, **13**, 138 (1940); MOORE, T., *Biochem. J.*, **34**, 1321 (1940)

196. SMITH, M. C., AND SPECTOR, H., *Ariz. Agr. Expt. Sta., Tech. Bull.*, **84**, 373 (1940)

197. BARRIE, M. M. O., *Biochem. J.*, **32**, 1467 (1938)

198. BARRIE, M. M. O., *J. Obstet. Gynaecol. Brit. Empire*, **46**, 49 (1939)

199. BARRIE, M. M. O., *Biochem. J.*, **32**, 2134 (1938); MARTIN, A. J. P., AND MOORE, T., *J. Hyg.*, **39**, 643 (1939)

200. UNDERBJERG, G. K. L., THOMAS, B. H., AND CANNON, C. Y., *Proc. Am. Soc. Animal Production*, **31**, 62 (1938)

201. Bryan, W. L., and Mason, K. E., *Am. J. Physiol.,* **131**, 263 (1940)
202. Pappenheimer, A. M., Goettsch, M., and Jungherr, E., *Conn. (Storrs) Agr. Expt. Sta., Bull.,* 229 (1939); Bird, H. R., and Culton, T. G., *Proc. Soc. Exptl. Biol. Med.,* **44**, 543 (1940)
203. Dam, H., and Glavind, J., *Naturwissenschaften,* **28**, 207 (1940)
204. Kudryashov, B. A., *Uspekhi Sovremennoĭ Biol.,* **6**, 318 (1937); *Bull. biol. méd. exptl. U.R.S.S.,* **6**, 220 (1938)
205. Carruthers, C., *Proc. Soc. Exptl. Biol. Med.,* **41**, 336 (1939)
206. Auchincloss, R., and Haagensen, C. D., *Proc. Soc. Exptl. Biol. Med.,* **42**, 703 (1939); Rider, D. L., *Am. J. Cancer,* **38**, 275 (1940)
207. Carruthers, C., *Am. J. Cancer,* **35**, 546 (1939)
208. Bryan, W. L., and Mason, K. E., *Proc. Soc. Exptl. Biol. Med.,* **43**, 375 (1940)
209. Euler, B. v., and Euler, H. v., *Z. physiol. Chem.,* **264**, 141 (1940)
210. Anderson, H. D., Elvehjem, C. A., and Gonce, J. E., *Proc. Soc. Exptl. Biol. Med.,* **42**, 750 (1939)
211. Morris, S. G., *Science,* **90**, 424 (1939)
212. Mackenzie, C. G., and McCollum, E. V., *J. Nutrition,* **19**, 345 (1940)
213. Shimotori, N., Emerson, G. A., and Evans, H. M., *J. Nutrition,* **19**, 547 (1940)
214. Knowlton, G. C., Hines, H. M., and Brinkhous, K. M., *Proc. Soc. Exptl. Biol. Med.,* **42**, 804 (1939); Evans, H. M., and Emerson, G. A., *Proc. Soc. Exptl. Biol. Med.,* **44**, 636 (1940)
215. Pappenheimer, A. M., *Proc. Soc. Exptl. Biol. Med.,* **45**, 457 (1940)
216. Telford, I. R., Emerson, G. A., and Evans, H. M., *Proc. Soc. Exptl. Biol. Med.,* **41**, 291 (1939)
217. Mackenzie, C. G., Mackenzie, J. B., and McCollum, E. V., *Proc. Soc. Exptl. Biol. Med.,* **44**, 95 (1940)
218. Mackenzie, C. G., Levine, M. D., and McCollum, E. V., *J. Nutrition,* **20**, 399 (1940)
219. Eppstein, S. E., and Morgulis, S., *Proc. Soc. Exptl. Biol. Med.,* **45**, 715 (1940)
220. Knowlton, G. C., and Hines, H. M., *Proc. Soc. Exptl. Biol. Med.,* **38**, 665 (1938)
221. Friedman, I., and Mattill, H. A., *Am. J. Physiol.,* **131**, 595 (1941)
222. Pappenheimer, A. M., *J. Mt. Sinai Hosp., N. Y.,* **7**, 65 (1940)
223. Telford, I. R., Emerson, G. A., and Evans, H. M., *Proc. Soc. Exptl. Biol. Med.,* **45**, 135 (1940)
224. Ekblad, M., and Wohlfart, G., *Z. ges. Neurol. Psychiat.,* **168**, 144 (1940)
225. Pappenheimer, A. M., and Goettsch, M., *Proc. Soc. Exptl. Biol. Med.,* **43**, 313 (1940)
226. Milhorat, A. T., Weber, F. C., and Toscani, V., *Proc. Soc. Exptl. Biol. Med.,* **43**, 470 (1940); Bicknell, F., *Lancet,* **I**, 10 (1940)
227. Wechsler, I. S., *J. Am. Med. Assoc.,* **114**, 948 (1940); Spies, T. D., and Vilter, R. W., *Southern Med. J.,* **33**, 663 (1940)
228. Bacharach, A. L., *Brit. Med. J.,* **I**, 890 (1940); Vogt-Møller, D., *Acta Obstet. Gynecol. Scand.,* **20**, 85 (1940)

229. Report of Referee, *J. Am. Med. Assoc.*, **114**, 2214 (1940)
230. Shute, E., *Am. J. Obstet. Gynecol.*, **35**, 970 (1938)
231. Cuthbertson, W. F. J., and Drummond, J. C., *Biochem. J.*, **33**, 1621 (1939)
232. Demole, V., *Z. Vitaminforsch.*, **8**, 338 (1939)
233. Smith, L. I., *Chem. Rev.*, **27**, 287 (1940)
234. Evans, H. M., *J. Am. Dietet. Assoc.*, **15**, 869 (1939); *J. Mt. Sinai Hosp., N. Y.*, **6**, 233 (1940)
235. British Nutrition Panel Symposium, *Vitamin E, A Symposium* (Heffer and Sons, London, 1939)

VITAMIN K

236. Fieser, L. F., *J. Am. Chem. Soc.*, **61**, 3467 (1939); Klose, A. A., and Almquist, H. J., *J. Biol. Chem.*, **132**, 469 (1940)
237. Fieser, L. F., Campbell, W. P., Fry, E. M., and Gates, Jr., M. D., *J. Am. Chem. Soc.*, **61**, 3216 (1939)
238. Fieser, L. F., Tishler, M., and Sampson, W. L., *J. Am. Chem. Soc.*, **62**, 996 (1940); Fieser, L. F., Tishler, M., and Wendler, N. L., *J. Am. Chem. Soc.*, **62**, 2861 (1940)
239. Tishler, M., Fieser, L. F., and Wendler, N. L., *J. Am. Chem. Soc.*, **62**, 1982 (1940)
240. Tishler, M., Fieser, L. F., and Sampson, W. L., *J. Am. Chem. Soc.*, **62**, 1881 (1940)
241. Fieser, L. F., *J. Biol. Chem.*, **133**, 391 (1940)
242. Karrer, P., Geiger, A., Rügger, A., and Salomon, H., *Helv. Chim. Acta*, **22**, 1513 (1939); Karrer, P., Geiger, A., Rügger, A., and Schwab, G., *Helv. Chim. Acta*, **23**, 585 (1940); Karrer, P., Epprecht, A., and König, H., *Helv. Chim. Acta*, **23**, 272 (1940)
243. Fieser, L. F., Tishler, M., and Sampson, W. L., *J. Am. Chem. Soc.*, **62**, 1628 (1940); Tishler, M., Fieser, L. F., and Wendler, N. L., *J. Am. Chem. Soc.*, **62**, 2866 (1940)
244. Riegel, B., Smith, P. G., and Schweitzer, C. W., *J. Am. Chem. Soc.*, **62**, 992 (1940)
245. Binkley, S. B., McKee, R. W., Thayer, S. A., and Doisy, E. A., *J. Biol. Chem.*, **133**, 721 (1940)
246. Makino, K., and Morii, S., *Z. physiol. Chem.*, **263**, 80 (1940)
247. Ansbacher, S., Fernholz, E., and Dolliver, M. A., *Proc. Soc. Exptl. Biol. Med.*, **43**, 652 (1940)
248. Emmett, A. D., Kamm, O., and Sharp, E. A., *J. Biol. Chem.*, **133**, 285 (1940)
249. Butt, H. R., Snell, A. M., Osterberg, A. E., and Bollman, J. L., *Proc. Staff Meetings Mayo Clinic*, **15**, 69 (1940)
250. Fieser, L. F., and Fry, E. M., *J. Am. Chem. Soc.*, **62**, 228 (1940); Foster, R. H. K., Lee, J., and Solmssen, U. V., *J. Am. Chem. Soc.*, **62**, 453 (1940); Lee, J., Solmssen, U. V., Steyermark, A., and Foster, R. H. K., *Proc. Soc. Exptl. Biol. Med.*, **45**, 407 (1940); Richert, D., Thayer, S. A., McKee, R. W., Binkley, S. B., and Doisy, E. A., *Proc. Soc. Exptl. Biol. Med.*, **44**, 601 (1940)

251. Dam, H., Glavind, J., and Karrer, P., *Helv. Chim. Acta,* 23, 224 (1940)
252. Koller, F., and Fiechter, N., *Schweiz, med. Wochschr.,* 70, 136 (1940)
253. Warner, E. D., and Flynn, J. E., *Proc. Soc. Exptl. Biol. Med.,* 44, 607 (1940); Smith, H. P., and Owen, C. A., *J. Biol. Chem.,* 134, 783 (1940)
254. Dam, H., and Glavind, J., *Z. Vitaminforsch.,* 10, 71 (1939)
255. Molitor, H., and Robinson, H. J., *Proc. Soc. Exptl. Biol. Med.,* 43, 125 (1940); Foster, R. H. K., *Proc. Soc. Exptl. Biol. Med.,* 45, 412 (1940)
256. Cohn, E. T., and Schmidt, C. L. A., *Proc. Soc. Exptl. Biol. Med.,* 41, 443 (1939)
257. Lozinski, E., and Gottlieb, R., *J. Biol. Chem.,* 133, 635 (1940)
258. Elliott, M. C., Isaacs, B., and Ivy, A. C., *Proc. Soc. Exptl. Biol. Med.,* 43, 240 (1940)
259. Almquist, H. J., and Zander, D., *Proc. Soc. Exptl. Biol. Med.,* 45, 303 (1940)
260. Tidrick, R. T., Joyce, F. T., and Smith, H. P., *Proc. Soc. Exptl. Biol. Med.,* 42, 853 (1939); Thayer, S. A., McKee, R. W., Binkley, S. B., and Doisy, E. A., *Proc. Soc. Exptl. Biol. Med.,* 44, 585 (1940)
261. Almquist, H. J., and Klose, A. A., *Proc. Soc. Exptl. Biol. Med.,* 45, 55 (1940)
262. Hepding, L., and Moll, T., *E. Merck's Jahresber.,* 53, 5 (1939)
263. Ansbacher, S., *J. Nutrition,* 21, 1 (1941)
264. Ansbacher, S., Fernholz, E., and Dolliver, M. A., *J. Am. Chem. Soc.,* 62, 155 (1940)
265. Fernholz, E., Ansbacher, S., and MacPhillamy, H. B., *J. Am. Chem. Soc.,* 62, 430 (1940)
266. Cheney, G., *Am. J. Med. Sci.,* 200, 327 (1940)
267. Ansbacher, S., *Proc. Soc. Exptl. Biol. Med.,* 44, 248 (1940)
268. McElroy, L. W., and Goss, H., *J. Nutrition,* 20, 527 (1940)
269. Woolley, D. W., and McCarter, J. R., *Proc. Soc. Exptl. Biol. Med.,* 45, 357 (1940)
270. Dam, H., Glavind, J., and Nielsen, N., *Z. physiol. Chem.,* 265, 80 (1940)
271. Brinkhous, K. M., and Warner, E. D., *Proc. Soc. Exptl. Biol. Med.,* 44, 609 (1940); Bollman, J. L., Butt, H. R., and Snell, A. M., *J. Am. Med. Assoc.,* 115, 1087 (1940)
272. Lord, Jr., J. W., Andrus, W. de W., and Moore, R. A., *Arch. Surg.,* 41, 585 (1940)
273. Hellman, L. M., and Shettles, L. B., *Bull. Johns Hopkins Hosp.,* 65, 138 (1939)
274. Wöhlisch, E., *Ergeb. Physiol. biol. Chem. exptl. Pharmakol.,* 43, 175 (1940); Riegel, B., *Ergeb. Physiol. biol. Chem. exptl. Pharmakol.,* 43, 133 (1940); Grossman, A. M., *J. Pediat.,* 16, 239 (1940); Brinkhous, K. M., *Medicine,* 19, 329 (1940)

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NUTRITION

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Each year's advance in knowledge has increased our understanding of the relation of nutrition to health, and our appreciation of the importance of dietary adequacy to good nutrition. Within recent years and especially during 1940, this subject has challenged the interest of a much larger group than the teachers and research workers in the field of nutrition. It has been prominent in the year's programs of professional and scientific societies differing widely in their fields of specialization. It has been featured in conferences of more general interest; it has been the mainspring of government programs of action for improving diets. Also in the latter half of 1940 the problems of national defense did much to make the general public conscious of the importance of a high level of nutrition to national preparedness. In consequence, the year saw an unusual volume of scientific and professional literature coming from the press as well as a large number of books, magazine articles, and newspaper stories on food and nutrition.

This review does not deal with the scientific research that has pushed back the frontiers of fundamental knowledge in biochemistry. Instead, it places chief emphasis upon attempts to evaluate the nutritional status of the population of the United States, and upon problems involved in translating present knowledge into action for betterment in nutrition. The heightened public interest in this matter brings a real challenge to scientists as well as to administrators to so guide the programs for improving nutrition that they will be permanently recognized as a part of national policy.

The state of the nutrition of our population has long been a topic of investigation. That there is considerable malnutrition has been the general conclusion of every study by whatever method and wherever conducted. An accurate appraisal is still lacking because of the difficulty in locating borderline cases—persons not suffering from obvious malnutrition but who have a nutritional impairment which deprives them of abounding health and vitality. Such an appraisal requires precise clinical and laboratory tests for specific nutritional deficiencies as well as thorough dietary studies. During the last few years a number of chemical and functional tests have been developed

or suggested. Some of the more promising ones are being tested, not only as methods in themselves, but for their applicability to the problem of determining the prevalence of specific nutritional deficiencies among large population groups.

The components of a satisfactory method for assessing the nutrition of populations were outlined by Youmans (1) in a symposium conducted by the American Institute of Nutrition in March 1940. He included an individual record of food consumption, full medical history and examination by competent physicians, and suitable laboratory tests for mild or early deficiencies of vitamins A, B, C, and D, nicotinic acid, protein, iron, and calcium, in so far as means for their detection are possible. He also pointed out that sufficiently large groups should be examined in order to satisfy statistical requirements and yet insure careful work. The methods being used in a medical evaluation of the nutritional status of a large group of high school students were reported in detail by Kruse et al. (2) in July 1940. This study was begun in 1938 by the Cornell University Medical College, the Milbank Memorial Fund, the New York City Department of Health, and the United States Public Health Service, with the assistance of the Work Projects Administration. Some two dozen examinations or tests are being applied in an effort to select those which give specific, dependable results and to determine criteria that may be used to evaluate the findings.

Few studies have been reported to date that have considered with equal thoroughness both the quantitative aspects of a person's diet and the specific clinical and laboratory tests designed to evaluate his nutritive state. Such studies are time-consuming and expensive to conduct, and the interpretation of findings often is a discouraging task because results are complicated by environmental and other factors which can be controlled only with difficulty, if at all. Between late 1939 and early 1941 some data have been reported from laboratory, clinical, and field studies of the responses of human beings to dietary treatment; of chemical analyses of body fluids and physical measurements of function or structure, sometimes but not always accompanied by an evaluation of diets; and of estimates of the nutritive values of diets of various population groups. Some of these findings, mostly preliminary or progress reports of current research, are summarized in the pages that follow.

Both the 17th and 18th annual conferences of the Milbank Memorial Fund (3, 4) gave attention to investigations of the nutrition

of various population groups. At the 18th annual conference, held in April 1940, preliminary reports from the New York City study, mentioned above, were given; these compared the nutritive status of children from low-income and high-income families with respect to vitamin A, vitamin C, and hemoglobin. The higher-income group consisted of students in a private school and the lower-income group, of students in a high school situated in the Lower East Side of the city. A large percentage of the latter were children of foreign-born parents, predominantly Jewish and Italian. Weekly incomes below $15 were reported by 12 per cent of the families of the first one thousand pupils examined in the Lower East Side school, and weekly incomes of $40 or more were reported by only 18 per cent.

In discussing the findings (4), Palmer stated that although the results of an adaptometer test showed a small significant difference in favor of the children from the more well-to-do families, little evidence was obtained that pointed conclusively to a frequent occurrence of vitamin-A deficiency among the poorer children. Wiehl reported very few cases of marked anemia among nine hundred pupils of the low-income group, but emphasized the need for more careful evaluation of standards for the age group under study. The most striking difference reported between children from lower- and higher-income groups was in the ascorbic acid content of the blood plasma. Kruse stated that 43 per cent of the boys and 31 per cent of the girls in the lower-income group had ascorbic acid values below 0.6 mg. per cent. Values equally low were found among only 6 per cent of the boys and 1 per cent of the girls in the higher-income group. No children in the higher-income group had ascorbic acid values below 0.2 mg. per cent, whereas 11 per cent of the boys and 5 per cent of the girls in the lower-income group had values this low.

The differences noted in the nutritive status of children of these economic groups are in harmony with the findings of food consumption studies made by Federal agencies (5, 6) that in cities the increase in consumption at successively higher income levels is relatively greater for fresh fruits than for any other major class of foods, and the increase in nutrients is relatively greater for vitamin C than for other dietary essentials.

Crane, Woods, Waters & Murphy (7) determined the level of ascorbic acid in the blood plasma of eighty-six rural children in grade schools in a northern Maine village in the autumn and again in the spring. The plasma of only two of the eighty-six children contained

as much as 0.8 mg. per cent of ascorbic acid in both seasons and dietary studies indicated that probably not more than one child in seven was eating a good source of vitamin C once a day. In the autumn the ascorbic acid content of the plasma of 28 per cent of the children was below 0.4 mg. per cent; in the spring 55 per cent were in this category. An association between low ascorbic acid in plasma and an inflammation of the gums was observed. In the autumn 29 per cent of the children had inflamed gums but by spring the condition had grown worse and 51 per cent had inflamed gums. Administration of ascorbic acid to 41 of the children with inflamed gums indicated that insufficiency of vitamin C probably was a factor in this condition; improvement followed within three weeks in the case of two thirds of the children treated.

Under the auspices of the Rockefeller Foundation, studies of nutritional status are being made in two rural communities in the South. One was undertaken in 1940 in a rural mill town in North Carolina, in co-operation with the Health Department and Duke University. The other, in co-operation with Vanderbilt University, was begun the year before in a rural area of Tennessee. At the 18th annual conference of the Milbank Memorial Fund Youmans (4) reported findings from the first 129 families studied in the latter investigation. Twenty-five per cent of 411 persons examined in a visual dark adaptation test gave values regarded as definitely abnormal, indicative of vitamin-A deficiency; 11 per cent of 502 persons tested had less than 0.3 mg. ascorbic acid per 100 ml. blood plasma, values considered to be unquestionably abnormal; and 26 per cent of 498 individuals examined had lower hemoglobin and 44 per cent lower red cell counts, than current minimum normal values for the sex and age groups studied. Evidence of protein deficiency was noted in 20 per cent of 455 individuals. Though incomplete and preliminary, these data indicate that a large proportion of the population included in the study is poorly nourished with respect to a number of essential nutrients. In addition to the cases noted above as definitely abnormal, an even larger proportion was regarded as border-line, probably subnormal, cases.

Minot and co-workers (8), in determining the ascorbic acid content of the serum of "normal" children (patients without serious or significant disease attending the pediatric clinic of Vanderbilt hospital), found that about half of those examined in the age range three to fifteen years had ascorbic acid values of 0.7 mg. or more per 100

ml. of serum. These values were considered indicative of a fairly satisfactory state of vitamin-C nutrition. However, of the entire group of 380 children under fifteen years of age examined, about 35 per cent of those tested during the winter and about 20 per cent tested during the summer had ascorbic acid values under 0.3 mg. per 100 ml. of blood serum; these values are considered indicative of serious deficiency. Less seasonal variation in values was found among children under three years of age than among those from three to fifteen years, a fact explained by the lesser variation in diets of the younger children. Relatively more sick than well children were found with serum low in ascorbic acid. Although the ascorbic acid levels of many children were low enough to be associated with a marked degree of deficiency of vitamin C in the tissues, a diagnosis of scurvy was rarely made, notwithstanding careful study of roentgenograms and a search for other clinical evidence. Clinical scurvy was not found even among children whose ascorbic acid levels were known to remain persistently below 0.2 mg. per 100 ml. of serum. The authors suggest that diets that are simultaneously deficient in calories and several other essential food factors may suppress the typical manifestations of deficiency; such low levels of ascorbic acid in otherwise adequately nourished persons might cause obvious scurvy. The investigators call attention to the lack of energy, poor appetite, mental apathy, and generally retarded development that characterized the children. These symptoms probably reflect not only a shortage of vitamin C, but diets that are inadequate in many respects.

Fox & Dangerfield (9) report observations on the effect of increasing the ascorbic acid content of diets of native mine workers in South Africa. Two groups of 950 each, matched with respect to tribe, home conditions, age, weight, physical fitness, and type of employment, were studied. The control group received a regular ration of the compound which afforded an average of 12 to 25 mg. of ascorbic acid per person per day; the experimental group was given in addition a special fruit drink each day that provided about 40 mg. of ascorbic acid. During the seven-month period of the study, twelve of the control group and one of the test group developed scurvy. No other significant difference between the two groups could be found in general health, in resistance to infection, or in physical efficiency. The investigators were unable to detect unfailingly, by careful clinical examination, a scorbutic condition and were impressed by the lack of correlation between their biochemical tests and clinical findings.

It is possible that more clear-cut findings might have resulted had the diets been adequate in all respects other than vitamin C.

A visual test for detecting vitamin-A deficiency given by Pett (10) to about one thousand students at the University of Alberta, showed approximately half to be deficient. Treatment with vitamin A of two hundred of the subjects classed as deficient resulted in a favorable change in their response to the test, and in many cases the recovery shown by the test was accompanied by improvement in the subject's sense of well-being. Pett's investigations indicate that adults are more likely to be deficient in vitamin A in February than in October. Of ninety single men on relief tested for visual adaptation, 64 per cent were considered to be deficient in vitamin A, as compared with about 50 per cent among nonrelief population groups examined. Both findings are not unexpected in view of a probable lower consumption of vegetables rich in vitamin A by the general population in that community in winter months than in fall, and of the generally poorer diet of relief groups as compared with higher-income nonrelief groups.

Widespread subclinical deficiency of thiamin seems probable in view of the large percentage of calories generally derived from highly refined food products, such as white flour and sugar. The recent experimental production of severe thiamin deficiency in humans by Jolliffe (11) and Williams (12) and their respective co-workers has made possible a more thorough study of the symptoms, manifestations, and treatment as applied to human nutrition than could be afforded from earlier clinical observation and animal experimentation.

As subjects Jolliffe used five young men, doctors at Bellevue Hospital, and Williams at the Mayo Foundation, ten young women, inmates of the Rochester State Hospital. Both groups of subjects received experimental diets adequate in all known essentials except thiamin. The diet of those at Bellevue Hospital furnished 0.473 mg. of thiamin per subject per day, and that of the women at Rochester only 0.15 mg. In both studies the urinary excretion of thiamin was determined, and close attention was given to the progressive development of deficiency symptoms and their disappearance when thiamin hydrochloride was added to the diet. Four of the five doctors studied at Bellevue Hospital developed symptoms of fatigue, lassitude, anorexia, precordial pain, burning of the feet, dyspnea on exertion, and palpitation within four to thirteen days, the time varying with the subject. Objective signs of skin hyperesthesia in the feet and ankles soon followed and changes in electrocardiograms were noted

in two of the subjects. The daily addition of 3.83 mg. of thiamin hydrochloride to the diet caused all subjective symptoms to disappear within three days and the objective signs within six days. The fifth subject failed to develop any symptoms even after thirty days on the diet low in thiamin.

Manifestations of deficiency in the ten women patients at the Rochester State Hospital appeared later than in the Bellevue group. The symptoms ultimately noted in all subjects after several weeks on the thiamin-deficient diet were fatigue, depressed mental states, weakness and dizziness, constipation, atony of muscles, backache, anorexia, nausea, loss of weight, and a slight roughness of the skin. The capacity to work, as measured by an exercising machine, fell progressively and electrocardiographic abnormalities developed. The rate of appearance and severity of the symptoms generally were augmented by physical activity and by cold and changeable weather; they developed later in the subjects studied from April to August than in those studied in the winter. Recovery was rapid and complete when thiamin hydrochloride was given. In four subjects the injection of a total of 4 mg. over a twelve-day period effected recovery while in four other subjects an initial injection of 1 mg. followed by fifteen daily injections of "small doses" (amount not stated) resulted in the disappearance of all symptoms and abnormalities. The remaining two subjects were given daily oral doses gradually increasing from 0.5 mg. to 2.0 mg. Of particular interest is the marked difference noted in these last two subjects when they were receiving 2 mg. as compared with 0.95 mg. daily. On the lower level they were free from clinical symptoms of thiamin deficiency, but with the higher intake of 2 mg. they had a feeling of "unusual well-being associated with unusual stamina and enterprise and . . . . were strikingly more alert and attentive."

The quantitative differences in the results of these studies need not be viewed as contradictions. Further research may indicate that differences in season, sex, muscular activity, and pressure or strain of work may be among the factors which affect the human requirement for thiamin and so may account for the earlier appearance of the symptoms among the young men receiving 0.473 mg. thiamin daily than among the mental patients with an intake of only 0.15 mg. daily. Certainly the advantages of a dietary allowance of 2 mg. a day, as compared with 1 mg., is deserving of further study.

It appears from these investigations that a whole series of physi-

cal and nervous disturbances may result from a thiamin intake of less than one milligram a day. While extensive dietary studies seldom show intakes of thiamin as low as those used to induce severe deficiency, a large proportion of families in this country, particularly those whose expenditures for food are below average, have diets providing less than 1.5 mg. thiamin per person per day (5, 13, 14).

Dietary deficiencies in factors of the vitamin-B complex other than thiamin are evidenced by the occurrence of pellagra, especially in the South, and by other suboptimal or pathological states which appear amenable to therapy with some form or portion of the complex. Demonstration of clear-cut relationships, however, requires further investigation.

The nutritional status of 428 school children in an industrial city in Pennsylvania has been reported upon by Zayaz, Mack, Sprague & Bauman (15). In addition to three reports for each child on a day's food consumption, examinations were made of nutritional status (as judged by a physical examination), body build and weight status, skeletal status (three measures), dental status, slump (standing and sitting), plantar contact, hemoglobin value, response to a biophotometer test, and response to a capillary fragility test. When classed into five arbitrarily defined groups according to findings from each examination it was found that children were least likely to achieve the highest class with respect to nutritional status (as shown by a physical examination), dental status, mineral density of skeleton, hemoglobin, and "total integration" test of the biophotometer. They fell into the lowest class most frequently on account of relatively inferior skeletal status, dental status, and poor showing on biophotometer tests. Only 3 per cent of the children studied came from families with cash incomes of $2,500 and over; the others were almost equally divided between two income classes, under $1,000 and $1,000 to $2,500, an income distribution which was believed to be representative of the city. Nutritional status as determined by physical examination, skeletal development, hemoglobin, and dark adaptation appeared to be related to income and consequent dietary differences. As compared with children from families with incomes under $1,000, those from families with incomes of $2,500 and over consumed twice as much milk, three times as many servings of citrus fruit, and about twice as many servings of green-colored vegetables and of meat. On the other hand, more servings of potatoes and bread were eaten by the children from the lower-income families.

Socioeconomic status was found by Jenss (16) to influence rate of growth among native-born white girls six to seven years old living in New Haven. The average percentage gain in weight over a period of nineteen to twenty months of 204 girls was less for children whose families were in poor circumstances as judged by the receipt of assistance or crowding in the home than for children whose families were in better circumstances. Children with three or four grandparents born in Italy gained more slowly than those who had three or four grandparents born in America but the socioeconomic influence was reflected in each group, whether of Italian or American ancestry.

Undoubtedly diet was a factor in this difference in rate of growth, but it must be recognized that other factors associated with economic status also affect growth and nutritive condition of the children. These include sanitation and roominess of home and play space, school and recreational activities, medical and dental care, immunization, and finally the sense of security made possible by the absence of poverty.

An anemia responsive to iron therapy was shown by Davis (4) to be widespread in certain counties of Florida. Of 4,335 rural school children examined, 53 per cent were found to have less than 11.4 gm. of hemoglobin per 100 ml. of blood, and 10 per cent had less than 8.3 gm. of hemoglobin per 100 ml. The anemic condition was especially common among children subsisting largely on foods produced in districts where mineral deficiencies in the soil were obvious from the prevalence of salt sickness among cows pastured there. In such districts the proportion of children found to be anemic ranged from 52 to 96 per cent.

Further evidence of impaired health in the United States may be found in the dental conditions of the population. In a survey made by the U.S. Public Health Service in co-operation with the American Dental Association in 1933 and 1934 (17), 90 per cent of the 1,438,-318 elementary school children examined were found to be in need of treatment for some form of dental disease. Recent analysis of these records by Kaiser & East (18) show that the incidence of dental caries in permanent teeth of white children was 97.3 per hundred boys of six to eight years old as compared with 348.5 per hundred boys twelve to fourteen years old. Comparable data for girls in the two age groups were 110.0 and 384.0 cavities per hundred children, respectively. In each age group the girls were found to have significantly more caries than the boys.

Publications within the last year indicate that the cause of dental

decay is still a controversial question. Jay (19) in summarizing the results of selected studies concludes:

Dental caries is not a manifestation of malnutrition and . . . . it cannot be controlled by adding minerals and vitamins to high carbohydrate diets . . . . control of carbohydrate consumption is the only dietary procedure thus far demonstrated by which the disease can be controlled.

Boyd (20) considers it necessary to recognize the importance of metabolic factors rather than a single nutritional component in any explanation of dental disease. After making a statistical study of the records of 250 children observed during the period 1926 to 1937 at the Children's Hospital, University of Iowa, he reported that children under suitable dietary control demonstrated a preponderant tendency to develop sclerosis of exposed dentin in carious areas soon after the dietary regimen was established and showed no further progress of decay as long as the regimen was carefully followed. Some of these controlled diets had as much as 60 per cent of their calories furnished by carbohydrate but usually the average was nearer 50 per cent. The most consistent characteristic of the controlled diets was their high content of protective foods, milk, eggs, vegetables, fruit, and cod liver oil. Boyd concludes:

The child with active caries must be considered to have a disturbance of metabolism, and this can be corrected most effectively through enrichment of the diet so that it will favor an optimum state of nutrition. Such treatment is conservative. It cannot lead to harm if carried out with insight, and it conserves not only the teeth but also their possessor.

Regardless of its etiology, dental impairment is to be prevented when possible and treated where it already exists. Tooth structure, which is initiated long before an individual's food habits and dental care come into the picture, is one of the determining factors in tooth health. The tooth-building materials, calcium, phosphorus, vitamin D, and vitamin C, together with vitamin A, which is essential to the normal functioning of the epithelial tissue from which the enamel-forming cells originate, need to be furnished early in prenatal life. Normal structure and maintenance of good health of all parts of the body are an undisputed objective and to this end adequate diets that include liberal quantities of protective foods and only moderate amounts of carbohydrate are indispensable for both mother and child. Nutrition programs, therefore, can proceed even though the har-

monizing of divergent opinions regarding the cause of dental caries must await further research.

Although it is difficult in human experience to clearly demonstrate the role of diet in health because so many environmental factors besides food are at work, it is often possible to indicate the effectiveness of improved diets in keeping at a high level the body's ability to resist or overcome disease. The fruitfulness of effort in this direction is shown by Tissue (21) in a recent study. Discovering deficiencies in vitamin A and C to be more prevalent in diets of children with tuberculosis than in those of healthy children studied as controls, one thousand tuberculous children were placed on a well-fortified dietary regimen. Ninety-two per cent showed improvement, but failure to adhere to such a program usually was followed by a recurrence of symptoms.

Numerous small-scale dietary studies made throughout the country indicate that present-day food habits are far from satisfactory. Thus, when one-day dietaries of eighteen boys and eighteen girls in each of nine counties in Tennessee were scored by Reynolds (22), 90 per cent were judged to be poor, with little use of milk, whole grain cereals, fresh fruit or vegetables. Clayton's study of the food habits and nutritional status of children in four different communities in Maine (23) indicates that the most outstanding nutritional defects were those resulting from dietary deficiencies in calcium, iron, vitamin A value, thiamin, ascorbic acid, and vitamin D. In a study by the inventory method of diets of fifty Pasadena families during a two-week period during the summer of 1938, Borsook & Halverson (24) found that 40 per cent of the families received less than 660 mg. calcium per person per day, 80 per cent less than 4,000 I. U. of vitamin A, and 33 per cent obtained less than 500 I. U. of thiamin. Dietary adequacy was related to food expenditure and most families spending less than 10 cents a person a meal ($2.08 per person per week) were found to have inadequate diets, while nearly all those spending more than this amount had adequate ones. Similarly, in New York City, where a study of malnutrition was used as a teaching device for the Public Health staff, Wheatley (25) reported that three fourths of the 5,593 children whom the school physician diagnosed as malnourished were found by home visits to be from low-income families.

In 1940 there came from the press the first of three volumes that present the detailed information on food consumption that was obtained in a nation-wide co-operative study undertaken in 1936 by five

Federal agencies—the Department of Agriculture, the Department of Labor, the National Resources Planning Board, the Central Statistical Board, and the Work Projects Administration (14). These data are but one segment of a study designed to provide comprehensive information on the way in which families in this country earn and spend their incomes. The facts were obtained from nonrelief native-born families in Chicago, New York, 6 other large cities, 14 middle-sized cities, 29 small cities, 140 villages, and 66 farm counties located in various parts of the country. Average expenditures for food, money value of food produced for family consumption, and quantities consumed of various groups of food are presented for families classified by region, the degree of urbanization of the community in which the family lived, family income, and family composition. Two of the volumes include an analysis of the nutritive value of the diets and classification of diets into good, fair, or poor on this basis. Some of the findings that bear upon the topic of the nutrition of our population are summarized in the following paragraphs.

The relation between income and expenditures for food is clear-cut. Among nonrelief village and city families with incomes under $500 a year, expenditures for food averaged about $1.25 per capita per week; the averages were $1.75, among those with incomes in the class $500 to $999; $2.75, for those with incomes in the class $1,500 to $1,999, and $3.80 for those with incomes between $3,000 and $5,000 a year. Although expenditures for food by the more well-to-do families were higher than among the less prosperous, the proportion of the money spent for current living that was used for food decreased with income. Thus, in the Middle Atlantic and North Central region, village families consisting of husband and wife spent 42 per cent of their money for living for food when incomes were in the class $250 to $499, but only 22 per cent when incomes were in the class $2,500 to $2,999.

Expenditures for food increased with the size of family in every income class, but the increases generally were insufficient to maintain the larger families at as costly a dietary level as that maintained by smaller families. Incomes needed by the larger families to maintain dietary levels comparable to those of small families were shown by comparisons made on an adult food-expenditure-unit basis. In the Middle Atlantic and North Central region, for example, village families of two—husband and wife—with incomes in the class $500 to $749, had food valued at an average of 10.7 cents per unit per meal

whereas families including parents and three or four children under sixteen years of age had meals which cost this much per unit only when incomes reached or exceeded $1,250 to $1,499.

The money value of the food of farm families usually represented a larger share of the value of family living than that of village or city dwellers at comparable income levels. This was due chiefly to the food-production programs of farm families. Home-grown products of white farm operators' families in the income class $1,000 to $1,249 represented from 44 to 65 per cent of the value of food in nine of thirteen farm sections studied. To supplement these farm-furnished foods, farm families spent for food a large share of the cash available for day-by-day living; in the income class mentioned, from 26 to 39 per cent of total money outlays for family living were spent for food in the 13 farm sections studied.

Two groups of food—vegetables and fruit, and meat, poultry, and fish—competed for first place in the expenditures for food eaten at home in villages and small cities in all sections of the country. At every income level each of these food groups took, as a rule, from a fifth to a fourth of the money expended for food. Milk, cheese, and cream combined, and grain products accounted for the next largest shares, about a sixth each. As incomes rose, a decreasing proportion was spent for grain products and fats, a decreasing or unchanging proportion for sugars and, usually, an increasing proportion for dairy products and meat. Food-spending patterns differed only slightly with size and composition of family. Greater differences in budgeting for food than now exist between income and family-type groups are necessary if families in this country are to secure best returns in food value for given expenditures for food.

In order to judge the nutritive qualities of the diets, food records for one week were obtained in connection with these studies of expenditures. A detailed inventory method was used for securing the records. A trained worker assisted with inventories and supervised the daily weighing of the foods and the daily recording of the age and work of every person eating from the family larder. From these data were determined the quantities of each kind of food consumed by the household. The nutritive value of the diet was computed from average figures on food composition. In interpreting the facts from this analysis it is necessary to keep in mind that information on the minerals and vitamins in many foods is still tentative, and that many of the values used in the computations tend to be high because losses

due to storage and cooking are not included. Furthermore, the potential nutritive value of foods brought into the house for family meals is higher than the value of food actually eaten. It was not feasible in so extensive a study to obtain from families the information needed to make adjustments for household waste of edible food.

In general, diets of low money value were found to be of poor nutritive quality in every community studied. In Middle Atlantic and North Central villages, for example, diets valued in the range $1.38 to $2.07 per week per food-expenditure unit, i.e., costing 20 to 30 cents per person per day, furnished the following average nutritive values per day per nutrition unit (equivalent to the needs of a moderately active man of 70 kg.) : 2,610 kcal.; 61 gm. of protein; 0.47 gm. of calcium; 1.08 gm. of phosphorus; 11.7 mg. of iron; 4,000 I.U. of vitamin A value; 1.2 mg. of thiamin; 40 mg. of ascorbic acid; and 1.4 mg. of riboflavin. All of these figures are below the allowances believed desirable for good nutrition. The most widespread deficiency probably occurred in calcium. Usually over 40 per cent and sometimes as many as 70 per cent of the families that spent 20 to 30 cents per person per day for food had diets that furnished less than 0.45 gm. of calcium per nutrition unit per day.

At a higher level of money value ($2.08 to $2.76 per week per food-expenditure unit), the average nutritive values of diets of groups of families were such as to suggest a fairly satisfactory although not generous food supply. The values for individual families within these groups, however, left much to be desired. For example, in the Middle Atlantic and North Central region, 66 per cent of the village families reported diets that provided less than 0.68 gm. of calcium per nutrition unit per day; 54 per cent, diets that furnished less than 6,000 I.U. of vitamin A; 36 per cent, diets that contained less than 1.5 mg. of thiamin; and 48 per cent, diets that provided less than 1.8 mg. of riboflavin per nutrition unit per day. In small or in middle-sized and large cities in the same region, corresponding proportions were even higher.

As village and city families had more money to spend for food, the foods they bought provided increased quantities of every nutrient considered, although the rate of increase was not the same for all nutrients. It tended to be least for calories and greatest for ascorbic acid. This shows that per capita consumption of inexpensive sources of calories, such as grain products, potatoes, sugars, and fats other than butter, increased but little as food expenditures rose, whereas the per capita consumption of fresh fruit and vegetables increased markedly.

An attempt was made to grade the diets as good, fair, or poor, and thus to provide a composite picture of their quality with respect to several nutrients.[1]

Any such grading must, of course, be regarded as provisional or tentative. Criteria other than those used might have been selected that would have imposed higher or lower standards for each grade of diet, and thus have classified relatively more or fewer families in each category. Probably, however, most scientists working in the field would agree that any diet classed as poor by the specifications given could be improved to the advantage of human welfare, and that the lower limits of the definition for an excellent diet are modest with respect to a number of nutrients. Among village and city families so few of the diets reported could meet the specifications for the excellent diets that a separate category could not be maintained for this class; hence diets graded good and excellent were grouped together.

With the income distribution and food price levels similar to those prevailing in 1935–36 it seems probable that at any one period about one fourth of the families in the United States would be found to have good diets according to the specifications listed, more than a third fair diets, and another third or more poor diets (26). It should be remembered, however, that the diet records were kept only for a single week, and hence they do not reflect weekly and seasonal variations in the nutritive quality. Hence, the proportion of families with diets that are always in the good class may be much less, and those that would drop into the fair or poor class part of the time may be greater than the figures show. Increased food purchasing power would be likely to increase the proportion of families with diets graded good. Even without such changes, however, the proportion with good

[1] A diet was classed as poor if it failed to meet the following specifications per nutrition unit per day with respect to one or more nutrients: Protein, 50 gm.; calcium, 0.45 gm.; phosphorus, 0.88 gm.; iron, 10 mg.; vitamin A value, 3,000 I. U.; thiamin (vitamin B_1), 1.0 mg.; ascorbic acid (vitamin C), 30 mg.; riboflavin, 0.9 mg. It was classed as fair if it met or exceeded the quantities of each nutrient specified above, but by a margin of less than 50 per cent with respect to one or more nutrients; as good, if it provided a margin of at least 50 per cent beyond the specifications listed for each nutrient, but less than 100 per cent in the case of the vitamins. A diet was classed as excellent if it provided per nutrition unit per day, the following nutrients in at least the quantities listed: Protein, 75 gm.; calcium, 0.68 gm.; phosphorus, 1.32 gm.; iron, 15 mg.; vitamin A value, 6,000 I. U.; thiamin, 2.0 mg.; ascorbic acid, 60 mg.; riboflavin, 1.8 mg.

diets could be greatly increased if all families used their resources for food to the best advantage.

The percentage of families with good or with poor diets was about the same whether they lived in villages, in small cities, in middle-sized cities, or in large cities so long as their food was of equal money value on a food-expenditure-unit basis. Thus at a fairly usual level of money value of food ($2.08 to $2.76 per unit per week), a few families, not exceeding 11 per cent, in each type of community in the North and West had diets that were graded good, and about a third, diets that were considered poor. The others had diets of an intermediate or fair grade.

Comparatively more nonrelief families on farms than in villages or cities had diets that were graded good and fewer had poor diets. Well-planned programs of food production for home use enabled many to have diets rich in protective foods. Relatively more of the ill-fed both on farms and in cities were found in the lower-income classes than in the higher; more in the larger families than in the smaller; more in the Southeast than in the North and West; and proportionately more among negro than among white families.

Figures afforded by dietary studies made in connection with investigations of income and expenditures such as those just cited are useful to all who are concerned with the problems of putting the newer knowledge of nutrition into practice, inasmuch as they clearly show the economic barriers to improved nutrition that must be surmounted and the extent to which nutrition can be improved through education in food selection and preparation.

Progress in evaluating nutritional status requires still closer linking of laboratory research, clinical observation, and careful dietary studies of population groups. Except for gross deficiencies in diet that result in obvious clinical symptoms of disease, it is difficult to demonstrate a relation between the nutritive quality of a diet and the state of general well-being as indicated by the physical condition of an individual. It is also difficult except in extreme cases to correlate the chemical composition of body fluids with tests of physiological function or the results of clinical examination. Probably this is to be expected since experience with animals under controlled laboratory conditions indicates that the ill effects of relatively minor deficiencies as well as the cumulative effects of good feeding can be fully recognized only after a relatively long time, sometimes not until the second, third, or later generations. Nevertheless, all types of studies reported

during the year under review indicate that there is much room for improvement in diets and in the nutritional status of the population of the United States. Despite the fact that there is still much uncertainty regarding the significance of certain tests of nutritive status, that methods and techniques are as yet too poorly standardized to make it possible to compare the results of different studies, and that more precise methods must be evolved, enough work has been done to make clear the need for a nutrition program on a nation-wide scale.

Authorities in public health and medicine are recognizing more and more the significance of the difference between minimal and optimal dietary standards. Winslow, in looking ahead after review of a half century's work of the Massachusetts Public Health Association (27), expressed the belief that nutrition was the central public health problem of the future. He considers the minor deficiencies which result from prolonged suboptimal nutrition to be more serious than the results of gross shortages; the latter will cause obvious symptoms which will demand immediate attention, whereas the former are insidious and by remaining unnoticed for long periods may lead to irremediable organic lesions.

Surgeon General Parran of the United States Public Health Service (28) has pointed out that the first step in improving the nutrition of the population is to recognize it as a national problem. He considers subclinical or latent malnutrition to be the most serious part of the nutrition problem; its ultimate effects may be worse than ancient famines, as it places a heavy responsibility on communities for the support of sick and disabled individuals over long periods.

Some of the problems involved in raising the nutritional level of any large proportion of the people of the United States are discussed by specialists in the field of economics, agriculture, and nutrition in the 1939 and 1940 *Yearbook of Agriculture*. Several chapters in each volume indicate that the problem must be attacked on many fronts (29 to 34). We need greater appreciation on the part of the general public of the importance of good nutrition to health, wider dissemination of present knowledge of food and nutrition, and more skill in food buying and preparation. In addition the economic limitations to obtaining adequate diets must be dealt with.

Under the latter point the articles cited from the *Yearbook* discuss briefly the implications for improved nutrition of such factors as an increase in national income and a shift in its distribution with accompanying effects upon the quantity and kind of food purchased; the

possibilities of increases in home food production for family use; subsidies to supplies for low-income groups; and the possibilities of reduction in food prices through efficient production and marketing. Of interest in connection with the growing spread between prices that farmers receive for food and the prices consumers pay is the nationwide investigation of food prices announced (35) late in 1940 by the antitrust division of the United States Department of Justice. There is to be a probing into alleged restraints of trade by processors of food, commission merchants, organized produce exchanges and auctions, truckers, and wholesale and retail distributors for the purpose of eliminating violations of antitrust laws.

Insufficient food purchasing power among low-income groups and the gradual loss of foreign markets have resulted in the piling up or wastage of unmarketable food surpluses on farms. The public has become increasingly aware of the dilemma presented by these farm surpluses on the one hand and the ill-fed village and city families on the other. There are no surpluses of protective foods from the standpoint of the population's nutritional needs; these are foods in which many ordinary diets, and especially those of low-income groups, tend to be low.

If the average consumption of protective foods by all families in this country could be raised to the level of those whose present diets can be rated "good" from the standpoint of nutrition, large increases in national consumption would result. The increases would be approximately as follows: milk, 20 per cent; butter, 15; eggs, 35; tomatoes, citrus fruit, 70; leafy, green, and yellow vegetables, 100. These figures are not maximum, as many freely chosen "good" diets do not include as much of the protective foods as many nutritionists believe advisable. For example, internationally recognized experts on nutrition recommend that we double our average consumption of dairy products. Yet to raise consumption even by the quantities just indicated would imply fairly large increases in purchasing power.

Much more food would be consumed by the nation than at present even if the diets of only the most needy families were raised to the point of covering average minimum nutritive requirements, with little margin for safety. If families that now have less of the protective foods than the quantities specified in a plan for the economical diet [outlined in the 1939 *Yearbook of Agriculture* (36)] could increase their consumption to the level of that diet plan, the resulting increases in national consumption would be approximately as follows: milk,

10 per cent; butter, 10; tomatoes, citrus fruit, 10; leafy, green, and yellow vegetables, 80.

Deficits in consumption are found chiefly though not exclusively among low-income groups. Although considerable improvement in diets could be accomplished by individual families through their own efforts—through wise food selection, careful food preparation, and by suitable programs of home production especially in villages and on farms—it is clear that the resources of many families in the lower income groups often are inadequate to provide nutritionally satisfactory diets under present circumstances. Measures for improving diets through education, through increasing the purchasing power of low-income groups, and through increasing the over-all efficiency of the marketing structure are essentially of a long-time nature. Meanwhile, special programs have been devised to improve the diets of low-income groups and at the same time to enlarge the domestic market for farm products.

The Surplus Marketing Administration of the United States Department of Agriculture is helping to bridge the gap between "price depressing farm surpluses and consumer underconsumption" by means of (a) direct purchase of surpluses for distribution to needy families through State welfare agencies, and for use in free school lunches for underprivileged children; (b) distribution of designated agricultural surpluses to public-aid families through regular trade channels, under the Food Stamp Plan. According to the annual report of the Administrator for the fiscal year 1939–40 (37), slightly over $117,700,000 was spent in purchasing surplus farm products for direct distribution and school lunch uses. Commodities distributed through State welfare agencies during the year went to a monthly average of 11,000,000 needy persons, and were used to supply lunches to nearly 3,000,000 school children. The Food Stamp Plan was in its first year of operation. By the end of the 1940 fiscal year it had been expanded to include 83 areas and was serving about 1,500,000 persons.[2]

[2] In brief, the Food Stamp Plan operates as follows: Families certified by local agencies as eligible for participation may buy from four to six dollars' worth of orange stamps each month for each family member and receive in addition blue stamps—fifty cents worth for each dollar's worth of orange stamps bought. The stamps may be used at any food store—orange stamps for the purchase of any food; blue stamps, for foods designated as surplus by the Secretary of Agriculture. The official list of designated foods varies from time to time. The list for January 1941 included: Fresh grapefruit, oranges, cabbage, apples, pears, onions (except green onions), Irish potatoes, dried

During the calendar year 1940 the number of areas in which the stamp plan was operating reached 125 to 150.. In this period $44,100,000 worth of blue stamps were used by low-income families in the purchase of the following items :[3] eggs, 24.8 million dozen; butter, 21 million pounds; oranges and grapefruit, 960,000 boxes; apples, 686,000 bushels; other fresh fruits, 9 million pounds; dried fruit, 15 million pounds; potatoes, 1.1 million bushels; dry beans, 22 million pounds; tomatoes, 7.7 million pounds; leafy, green, and yellow vegetables, 24.6 million pounds; other vegetables, 14.7 million pounds; flour, 167 million pounds; other cereals, 55.7 million pounds; lard, 30.3 million pounds; and pork, 64 million pounds.

According to an economic analysis of the Food Stamp Plan based on results of the first year of its operation (38), it appears that purchases of food made with blue stamps account for 3 to 6 per cent of the total retail food sales in areas in which the stamp plan is in force, although in low-income sections of these areas, this additional purchasing power may account for as much as 10 per cent of the sales. These figures indicate that the Food Stamp Plan in its present scope, though useful in raising levels of food consumption of participants, is far from a panacea for eliminating underconsumption of protective foods in this country.

One chapter in the report just cited deals with the influence of the Food Stamp Plan on dietary patterns of low-income families as shown by a study made in Dayton, Ohio, in August and September, 1939. The chief difference found at that time between the consumption of participants and nonparticipants of comparable economic status lay in the significantly higher average consumption of butter and eggs by participants, and a tendency toward higher consumption of all other food groups, especially of fruit and vegetables other than potatoes. As a result, the nutritive value of the diets of participants tended to be more generous than those of nonparticipants in every respect, particularly in vitamin A.

Diets of low-income groups frequently are low in calcium—a nu-

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prunes, raisins, butter, lard, all pork (except that cooked or packed in metal or glass containers), dry beans, eggs, corn meal, hominy grits, rice, wheat flour, and whole wheat flour. Fresh carrots will be available during January in eleven Northeastern States in addition to the surplus foods obtainable nationally in all areas for that period.

[3] U.S. Department of Agriculture, Surplus Marketing Administration. Distribution and Purchase Division, mimeo. report January 25, 1941.

trient not provided in generous quantities by any of the foods recently
on the surplus list for direct or stamp plan distribution. For this rea-
son a special program sponsored by the Surplus Marketing Admin-
istration is of interest, whereby certain low-income groups may buy
milk for less than the usual retail prices. These arrangements have
been made in a number of cities, including Boston, Chicago, New
Orleans, New York, and Washington, D.C. The special prices are
made possible through marketing agreements in which farmers take a
price somewhat lower than that for class-I milk but higher than that
received for milk for manufacturing purposes, local dealers submit
competitive bids for processing, distribution usually is made with the
help of WPA labor, and some Federal subsidy is provided. In this
way milk that meets all of the usual local requirements for quality is
made available for family purchase, for purchase by school children,
and for free distribution to needy children receiving free lunches.

The extent of benefits derived by participants in these low-price
milk programs may be illustrated by a study made in 1940 in Wash-
ington, D.C. In this city, milk is available at 5 cents a quart to fami-
lies certified as receiving or eligible to receive public assistance or
certified as eligible for and awaiting assignment to WPA employment.
Families are permitted to purchase at this price one pint of milk daily
for each child (persons under seventeen years of age) and one half
pint daily for each adult. Nursing or expectant mothers, unattached
adults living alone, and adults with special dietary needs are permitted
to buy one pint daily. A study, still unpublished, made by the De-
partment of Agriculture of the quantity of milk consumed by identical
families before and after the inauguration of the low-price milk pro-
gram showed that white families participating in the plan increased
their consumption of fluid milk from 1.5 to 3.1 quarts per person per
week, and negro families from 0.9 to 2.8 quarts per person per week.

Another program that subsidizes consumption is the free school
lunch for needy and undernourished children. Foods on the surplus
list are donated by the Surplus Marketing Administration; local spon-
sors provide other needed foods, and help in preparation and service
is given by the Work Projects Administration. In March 1940,
according to a report of Southworth & Klayman (39), about 2.5 mil-
lion children in more than 35,000 schools were receiving between-
meal food or noon lunches at school made in part from surplus foods.
Plans for the current school year are to expand the program so as to
serve some 6 million needy children.

It is impossible to generalize on the net effect of these special programs upon the nutritive value of the diet of any family or group of families. The result will depend on the assortment of foods that are made available, the quantities of each used by the participants, and the period over which the food is available, as well as on the customary diet of the participants and the influence of the receipt of surplus products on usual food-spending habits. In some households and for some commodities surplus products can be and apparently are being consumed as net additions to quantities customarily purchased. This seems to be true for certain fruits and vegetables, milk in some forms, and to a lesser degree, for butter and eggs. For some other foods such as grain products this is not the case; with such products on the surplus list, usual purchases may be reduced somewhat (maintaining nevertheless some increase in total consumption) and the money thus released may be spent in part for other foods and in part for other items needed for current living.

Because food takes such a large share of the budget, it is generally in food expenditures that cuts are made when income drops or unusual outlays must be made. In August and September, 1939, when the city of Chicago was providing its relief clients with only 65 per cent of the city's standard budget, the staff of the Elizabeth McCormick Memorial Fund (40) found that a large proportion of the clients were spending for rent, fuel, and clothing money that the city had intended to be used for food. Sixty-one per cent of the 512 families whose expenditures were studied were found to be spending less than half as much money for food as was needed for a minimum adequate diet. Contrasted to this, 89 per cent paid all or part of their rent, with 64 per cent paying more than half again the amount allocated for this item in the official budget. The fear of eviction was greater than the fear of malnutrition and its consequences.

Ingenious as are some of the devices for subsidizing consumption, and necessary as is direct public assistance under present circumstances, everyone looks forward to a time when there can be adequate incomes for all. Every normal person wants to be productive enough to have command over the goods and services needed for wholesome and abundant living, including purchasing power sufficient to buy fully adequate diets.

To make it easier to have satisfactory diets with present spending habits much interest has centered around fortification of certain foods with added vitamins and minerals. Variance of opinion has been ex-

pressed as to the advisability of such a practice. Arguments against it include statements to the effect that comparatively little is known either of human requirements for these essentials or of the danger of excesses; that little is known of the stability of these substances in artificial combinations with other food constituents; that the practice would present many problems of expense and waste; and that it would lead to still greater indifference and lack of responsibility on the part of the consumer to choose food wisely. Arguments in favor of fortifying foods pass over these problems to lay stress on the importance of supplying special dietary essentials immediately and comparatively cheaply to all persons rather than prolonging the period during which some subsist on suboptimal diets while the slow processes of education in food selection and economic adjustments are taking effect.

The Council on Foods of the American Medical Association adopted (41) a general policy which recognizes the merit of restoring the important nutritional essentials which have been lost in processing to general purpose foods (foods which are ordinarily considered to be useful for any member of the family). In the case of cereals the policy operates as follows: The Council has seen no objections to the restorative addition of thiamin hydrochloride to cereal products to bring them up to the level of oatmeal, the cereal having the highest vitamin B_1 content (300 International Units per 100 gm. of dry material). The Council takes the same position with respect to the addition of other essentials such as riboflavin, but insists that the "reference" food be specifically stated.

The United States Food and Drug Administration began in August 1940 to hold hearings and to gather testimony from physicians, nutritionists, other scientists, millers, and laymen regarding the fortification of flour and bread as one possible and effective means of improving the nutrition of the Nation. Hearings are nearing completion; standards and a name for the new product probably will be announced early in 1941. Reinforcement of flour may be accomplished either by adding pure chemical substances such as thiamin hydrochloride and calcium and iron salts to white flour or by returning to the highly milled product certain of the "mill streams" which are particularly rich in the nutrients, or by a combination of these methods.

The obvious importance to national defense of having abundant supplies of food when and where needed both for military and civilian populations and the recognition of the relation of good nutrition to the health and morale of both groups has led the National Defense

Advisory Commission to request that these problems be given consideration. The Agricultural Division of the National Defense Advisory Commission set up a committee in the fall of 1940 to undertake an inventory of the nation's food resources, including production potentialities, stocks, facilities for manufacturing and processing, food distribution, and food requirements. The President of the United States appointed a co-ordinator in late 1940 charged with the responsibility of enabling existing agencies concerned with health, welfare, and related activities to function as effectively as possible in contributing to a program of total defense. Nutrition was named as one of the fields in which advisory committees to the co-ordinator will serve on federal, regional, and local levels. The co-ordinator has announced that his policy will be to utilize services of existing agencies in so far as these are involved in the objectives of the national defense program; where present functions of agencies do not comprehend all of the services necessary for defense purposes, they are to be expanded so that the aggregate functions of existing agencies will meet defense needs that are within the public responsibility.

In connection with developing a national policy and program for nutrition in this country the British publication *Feeding the People in War-Time* by Orr & Lubbock (42) is pertinent. The authors believe that the progress in improving nutritional status in Great Britain can be continued in war as well as in peacetime through proper government subsidy, expansion and direction of farming activities, and price fixing or rationing of important foodstuffs. Although many of the specific measures suggested for Britain are not directly applicable to circumstances in this country, their statement of general principles is of interest.

Before closing this review of progress and problems in nutrition in 1940, mention should be made of the conference on nutrition problems of the American republics held in Buenos Aires in October 1939 under the auspices of the League of Nations. (The report of the meeting has not appeared, due to the war situation in Europe where it was being printed.) Without exception, the representatives of the republics to our south stressed the prevalence of malnutrition in their populations, and named as contributing factors poverty, unsatisfactory transportation and refrigerating facilities, and lack of widespread information on food selection and preparation. One noteworthy event of the conference was the announcement by the Argentine government that it would establish scholarships for citizens of the various coun-

tries of this hemisphere, covering tuition and maintenance for a three-year period of study at the National Institute of Nutrition in Buenos Aires. This institute offers a three-year course at the university level for nutritionists and dietitians, and a two-year postgraduate course for young doctors after they have finished interneship. This gesture of goodwill on the part of Argentina can do much to help raise the nutritional level of the Americas. The urgent need for trained workers in the field was repeatedly indicated by representatives from Central and South America. In the last academic year three nations took advantage of the opportunity afforded by Argentina's generosity: Mexico sent three doctors; Brazil, two doctors and two young women; and Paraguay, three doctors and two young women. It is reported that several other Spanish-speaking countries intend to send students next year.

LITERATURE CITED

1. YOUMANS, J. B., *J. Nutrition,* 19, 21 (1940)
2. KRUSE, H. D., PALMER C. E., SCHMIDT, W., AND WIEHL, D. G., *Milbank Mem. Fund Quart.,* 18, 257 (1940)
3. PROCEEDINGS OF THE SEVENTEENTH ANNUAL CONFERENCE OF THE MILBANK MEMORIAL FUND, *Millbank Mem. Fund* (1939)
4. PROCEEDINGS OF THE EIGHTEENTH ANNUAL CONFERENCE OF THE MILBANK MEMORIAL FUND, *Milbank Mem. Fund* (1940)
5. STIEBELING, H. K., MONROE, D., PHIPARD, E. F., ADELSON, S., AND CLARK, F., *U.S. Dept. Agr. Misc. Pub.* (In press, 1941)
6. WILLIAMS, F. M., AND KAPLAN, A. D. H., *U.S. Dept. Labor Bull.,* No. 648 (1940)
7. CRANE, M. M., WOODS, P. W., WATERS, E. M., AND MURPHY, E. F., *J. Nutrition,* 19, 16 (1940)
8. MINOT. A. S., DODD, K., KELLER, M., AND FRANK, H., *J. Pediat.,* 16, 717 (1940)
9. FOX, F. W., AND DANGERFIELD, L. F., *Proc. Transvaal Mine Med. Officers' Assoc.,* 19, March (1940)
10. PETT, L. B., *J. Lab. Clin. Med.,* 25, 149 (1939)
11. JOLLIFFE, N., GOODHART, R., GENNIS, J., AND CLINE, J. K., *Am. J. Med. Sci.,* 198, 198 (1939)
12. WILLIAMS, R. D., MASON, H. L., WILDER, R. M., AND SMITH, B. F., *Arch. Internal Med.,* 66, 785 (1940)
13. STIEBELING, H. K., AND PHIPARD, E. F., *U.S. Dept. Agr. Dept. Circ.,* No. 507 (1939)
14. STIEBELING, H. K., MONROE, D., COONS, C. M., PHIPARD, E. F., AND CLARK, F., *U.S. Dept. Agr. Misc. Pub.,* No. 405 (1941)
15. ZAYAZ, S. L., MACK, P. B., SPRAGUE, P. K., AND BAUMAN, A. W., *Child Development,* 11, 1 (1940)

16. Jenss, R. M., *Human Biol.,* **12**, 532 (1940)
17. Messner, C. T., Gafafer, W. M., Cady, F. C., and Dean, H. T., *U.S. Pub. Health Bull.,* No. 226 (1936)
18. Kaiser, H., and East, B. R., *J. Am. Dental Assoc.,* **27**, 1430 (1940)
19. Jay, P., *J. Am. Dental Assoc.,* **27**, 393 (1940)
20. Boyd, J., *J. Am. Dental Assoc.,* **27**, 750 (1940)
21. Tissue, K. A., *J. Am. Dietet. Assoc.,* **16**, 313 (1940)
22. Reynolds, D., *Master's Thesis* (University of Tennessee, 1939)
23. Clayton, M. M., *Maine Expt. Sta. Bull.,* No. 4, 85 (1939)
24. Borsook, H., and Halverson, W. L., *Am. J. Pub. Health,* **30**, 895 (1940)
25. Wheatley, G. M., *Am. J. Pub. Health,* **30**, 542 (1940)
26. Stiebeling, H. K., *U.S. Dept. Agr. Misc. Pub.,* No. 430 (1941)
27. Winslow, C. E. A. *Am. J. Pub. Health,* **30**, 325 (1940)
28. Parran, T., *Tech. Rev.,* **42**, 323 (1940)
29. Howe, P. E., *U.S. Dept. Agr. Yearbook,* 131 (1939)
30. Stiebeling, H. K., *U.S. Dept. Agr. Yearbook,* 380 (1939)
31. Cavin, J. P., Stiebeling, H. K., and Farioletti, M., *U.S. Dept. Agr. Yearbook,* 329 (1940)
32. Hoffman, A. C., and Waugh, F. V., *U.S. Dept. Agr. Yearbook,* 627 (1940)
33. Perkins, M., *U.S. Dept. Agr. Yearbook,* 650 (1940)
34. Burtis, E. L., and Waugh, F. V., *U.S. Dept. Agr. Yearbook,* 656 (1940)
35. U.S. Department of Justice, *Public Statement* (Nov. 25, 1940)
36. Stiebeling, H. K., and Clark, F., *U.S. Dept. Agr. Yearbook,* 321 (1940)
37. U.S. Department of Agriculture, *Report of the Administrative Official in Charge of Surplus Removal and Marketing Agreement Programs* (1940)
38. Gold, N. L., Hoffman, A. C., and Waugh, F. V., *U.S. Dept. Agr., A Special Report,* (Oct., 1940)
39. Southworth, H. M., and Klayman, M. I., *Agr. Situation,* **24**, No. 8, 19 (1940)
40. A Study by the Elizabeth McCormick Memorial Fund with the Cooperation of Twenty-five Participating Agencies, Pamphlet *(Council of Social Agencies of Chicago,* 1940)
41. Council on Foods, *J. Am. Med. Assoc.,* **115**, 218 (1940)
42. Orr, J., and Lubbock, D., *Feeding the People in War-Time* (Macmillan and Co., London, 1940)

RELATION OF SOIL AND PLANT DEFICIENCIES AND OF TOXIC CONSTITUENTS IN SOILS TO ANIMAL NUTRITION

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Within the limits of the space allowed, it is the objective of this review to discuss those aspects of the general topic which particularly indicate the interrelationships between the nature of the soil, and plant growth, composition, and nutritive value. Primary attention is given to those nutrients for which the relations have been traced from the soil through the plant to the animal. The nutrition of grazing animals receives much consideration because here the animal depends solely upon the production of a given area and the relation to the soil is a direct one. The writer has examined some one hundred papers dealing with the vitamin content of plants as influenced by soil factors. This important phase of the general topic must be omitted because of space limitations.

The review is concerned with the influence of soil factors upon the nutritional quality of food crops rather than upon crop yields. In preparing the review it has become apparent that the agronomist has not recognized sufficiently the importance of yield of available nutrients as distinguished from dry-matter yields. The nutritionist, on the other hand, has been at fault in studying food crops without a sufficient appreciation of their variability as governed by soil and cultural factors and of the importance of taking these factors into account in many types of investigations. The manifold aspects of the topic here under review and their importance both to agriculture and to human and animal welfare have recently been appraised by Auchter (1). This article also presents a discussion of needed lines of investigation in this broad field. A very comprehensive survey of the literature on the composition of crops with particular reference to the soils on which they are grown has been prepared by Beeson (2).

Mineral Nutrients

It is clear that the most direct and evident relationship between the soil and animal nutrition exists in the case of the mineral elements. Nearly the same list is required for both plant and animal life. The agronomist is interested in the soil minerals as they influence plant growth and in the minerals in the crop in so far as they reflect plant needs and soil conditions. The animal nutritionist is primarily concerned with the relation of the mineral content of plants to their nutritive value, and he is particularly interested in those minerals which are likely to be deficient in terms of the animal's needs unless special attention is given to them in selecting the ration.

Phosphorus.—It is well understood that there are large areas in various countries throughout the world where serious losses among grazing animals occur because the forage is abnormally low in phosphorus due to soil conditions, including factors which are often aggravated by climatic variables. Unfortunately the specific soil relations have not been studied in many of the areas, but there are sufficient data to show that a variety of soil and cultural factors are concerned in various situations. A definite lack in the soil phosphorus supply is by no means the sole cause. In fact the low phosphorus content of the herbage appears to be due more frequently to soil conditions which render the phosphorus unavailable to the plant rather than to an absolute deficiency in the soil. Such is the situation in South Africa, where the pioneer studies of Theiler and associates (3) revealed the tremendous losses to the livestock industry which resulted from the very low phosphorus content of the pastures. The soil in the area in question contains only 0.0005 to 0.001 per cent of available phosphorus pentoxide as compared with a total content of 0.03 to 0.11 per cent. In this same general region a soil containing 0.009 per cent of available phosphorus pentoxide supports a pasture on which animals remain healthy.

In the areas in Tennessee recently studied by workers at the State Experiment Station (4, 5, 6), on the other hand, marked differences in total phosphorus content are apparently primary factors. In the Central Basin of Middle Tennessee there are extensive soil areas, developed from a phosphatic limestone, which are recognized as unusually fertile soils. Some of these are said to contain as much as 6,200 pounds of phosphorus pentoxide and 6,400 pounds of calcium oxide per acre to plow depth. In contrast, the Highland Rim surrounding this Basin consists largely of poor, nonphosphatic soil with

only 1,000 pounds of phosphorus pentoxide and 3,200 pounds of calcium oxide per acre. There is a marked difference in the phosphorus content of hay grown in certain soils in the two areas. For example, lespedeza cut in early bloom on Maury silt loam in the Basin contained 0.27 per cent phosphorus, whereas the same hay cut at the same stage from Clarksville silt loam in the poor areas contained only 0.10 per cent phosphorus.

Williams, MacLeod & Morrell (7), in experiments with rats, have compared the availability of the phosphorus in the Tennessee hays grown on good and poor soils. Lespedeza, alfalfa, soybean, and red clover hays were studied, all of which showed marked differences in phosphorus content according to the soil source. When high and low phosphorus hays of the same kind were introduced into diets otherwise adequate, in amounts which furnished the same phosphorus intakes, the diet containing the hay high in phosphorus proved superior in terms of growth and phosphorus stored. The results were apparently due to differences in the digestibility of the diets as a whole rather than to differences in the form of phosphorus or in its utilization after absorption. It would be interesting to repeat this study with a species which has a digestive tract more suitable for hay consumption than is the case with the rat. The Tennessee Experiment Station (5) has announced the results of feeding steers with clover hay from the Basin and from the Highland Rim; the animals fed the Basin hay made approximately 50 per cent better gains. The details of this experiment are not available.

In areas in Florida where phosphorus deficiency troubles have been reported (8) the phosphorus content of the forage is lower on sandy loams than on coarser soils. This content is correlated with the water-soluble phosphorus present in the soil, according to Bryan & Neal (9). The total phosphorus in the surface horizon of virgin soils is low (0.03 to 0.04 per cent) compared to cultivated, fertilized soils which run two to seven times higher, as indicated by the studies of Rogers and co-workers (10).

In some grazing areas where stock suffer severely, such as in Florida, it is apparent that the troubles are not due solely to the low phosphorus content of the forage. The yield of forage is low and its nutritive quality may be deficient in several respects. Multiple soil deficiencies are certainly involved in some cases, although phosphorus deficiency alone may reduce yield, lower digestibility, and perhaps affect other nutritive qualities unfavorably. In other areas where ani-

mal troubles are severe (11, 12) both legumes and grasses grow luxuriantly and apparently provide liberal amounts of nutrients other than phosphorus.

It is a common observation that phosphorus deficiency troubles are more severe in times of drought and that in borderline situations they may occur only in dry seasons or years. With a lower rainfall, the forage is lower in phosphorus, as is shown by data obtained in the Minnesota area (11). A recent report from Wyoming (13) indicates the importance of water relationships in areas where evident troubles are comparatively rare. The general finding that the phosphorus content of grasses and legumes grown on various soils is correlated with the amount of rainfall is illustrated by the studies of Daniel & Harper (14) and of Salgues (15).

There are many reports showing that the fertilization of soils in phosphorus-deficient areas increases the phosphorus content of the forage (4, 10, 11, 16) as has also been shown by the agronomist for more productive soils and for various crops. It is not sufficiently appreciated, however, that the extent of this influence depends upon many soil and cultural factors and that sometimes added phosphorus is fixed in the soil in an unavailable form, thus having little or no influence on either yield or phosphorus content. Among these factors are hydrogen ion concentration, moisture relations, calcium supply, nature and amount of organic matter, etc. Large amounts of iron and aluminum make phosphorus unavailable in acid soils, and calcium in a soil having a pH above 7 tends to produce a similar effect. McGeorge (17) has observed that in certain phosphorus-deficient areas of Arizona the soil alkalinity arising from calcium hydroxide and solid phase calcium carbonate may render the phosphorus unavailable to plants. There is some evidence that phosphorus-deficient areas may be the result of a too high calcium content where the pH is 7.5 or higher. Soil differences in the response to phosphate fertilizer are illustrated by the Tennessee studies (4). Phosphate fertilizer increased the phosphorus content of lespedeza by 40 per cent in one soil but by only 17 per cent in another. In the Florida area (9) it was reported that one soil, because of its high phosphorus-fixing power, required comparatively large applications of fertilizer to increase the phosphorus content of the forage.

Some recently studied areas of phosphorus deficiency in animals are those in British Guiana (18) where well-watered native grass is reported to contain only about 0.01 per cent of phosphorus. An intro-

duced grass contained approximately only 0.02 per cent in the "bad" area whereas it contained over 0.11 per cent when introduced into a normal area. Recent reports have also come from Louisiana (16) where large differences in the phosphorus content of hay from virgin and cultivated, fertilized soils have been noted. In Mississippi, where bone chewing is common, it has been found that the native grasses, broom sedge and carpet grass, may be as low as 0.04 per cent in phosphorus, in contrast to figures of 0.1 to 0.3 per cent in better soils. Interesting cases of phosphorus deficiency in horses, characterized by lameness and very low blood inorganic phosphate, have recently been reported from Kansas (19) and ascribed to drought conditions.

Cases of acute phosphorus deficiency in herbivorous animals are not limited to range or pasture feeding. They can occur, of course, in barn or lot feeding where the roughage has come from an area typically low in phosphorus. It is clear, on the other hand, that many of these troubles are more properly ascribed to poor feeding practices. There is a failure to recognize that even the best of roughage is not a rich source of phosphorus in terms of the animal's need, that certain concentrates are rich in the element and some are not, and that under conditions of rapid growth or high milk production special attention must be given to selecting feeds which will combine to make the ration adequate. These facts are brought out clearly in a recent investigation by Forbes & Johnson (20) of phosphorus deficiency among cattle in Pennsylvania. They found a number of situations where the animals were suffering from the deficiency as indicated by physical condition, blood inorganic phosphate level, and feed analyses. They found also that the roughage being fed, particularly corn stover, timothy hay, and wheat straw, was generally low in phosphorus. They did not find the phosphorus deficiency to be characteristic of any area in the state. They concluded that it should never be present with good feeding practices, particularly the substitution of better roughage for the straw and stover and the selection and feeding of concentrates to increase the phosphorus content of the ration. In some cases investigated it was evident that the animals simply were not getting enough to eat.

Many studies have shown that the phosphorus content of vegetables is markedly influenced by soil factors. Bishop (21) found that the phosphorus content of cabbage and lettuce grown in Alabama soils varied both with the type of soil and with fertilization. For example, with an addition of two hundred pounds of superphosphate per acre the phosphorus content was 0.009 per cent on Cecil soil and

0.017 per cent on Hartselle soil. With an addition of two thousand pounds of phosphate the figures were 0.026 per cent and 0.043 per cent respectively for the two soils. Davidson & LeClerc (22) studied five vegetables and found marked variations in phosphorus content in the same variety from different soils and with different fertilizer treatments. For example, the figures for spinach ranged from 0.81 to 1.01 per cent and for kale, 0.25 to 0.78 per cent, while for broccoli the differences were very small. Elmendorf & Pierce (23) have reviewed other literature, and presented data on the calcium and phosphorus content of corn, beans, beets, and cabbage fertilized with nitrogen, phosphorus, and potassium alone or in combination. The fertilizer treatments increased the phosphorus content in all vegetables.

Calcium.—In contrast to the situation for phosphorus, there are very few reports of area troubles in animals caused by a lack of calcium in the herbage. In the United States it appears that such an area exists on acid sandy soils in Florida, though specific soil studies have not been made. Becker and associates (24) have reported that grass forages grown on these soils contain relatively small amounts of calcium. Dairy cows dependent on these home-grown roughages withdrew mineral matter from their bones to the extent that they were easily broken. Both production and health were severely affected. That these troubles were due to a lack of calcium rather than of phosphorus was indicated by the fact that the phosphorus intake was greatly in excess of requirements as a result of the concentrates being fed. Mention has been made of the rather low calcium content in the grass in areas in Florida (8) where the phosphorus content was apparently the primary deficiency in the absence of supplementary feed. A lack of calcium may have been a contributing factor. In dairy feeding (24), where high phosphorus concentrates are included, the low calcium content of the forage becomes the main factor in the troubles noted.

Legume pasture or hay always contains enough calcium to meet the animals' needs, and thus variations here are of minor importance. In many soils, however, the calcium supply, the acidity, or other factors are such that legumes will not grow normally or even not at all. Grasses are much less affected by these factors. They are always much lower in calcium than are legumes and a satisfactory growth is no proof of an adequate calcium content. Generalizations regarding the adequacy of grass hay in calcium content for animal nutrition are unsafe because of the very large variations that can occur. In a study

by Hart and associates (25) in Wisconsin a "poor ration" based on timothy hay grown on acid soil was found entirely adequate for high milk production without mineral losses from the bones over several lactations, resulting in the conclusion that a ration with timothy hay as the roughage will provide satisfactory nutrition. The hay used ranged from 0.35 per cent to 0.5 per cent calcium. A similar report on the adequacy of timothy hay as a source of calcium has come from Pennsylvania (26) where the hay contained 0.36 per cent of the element. In New York State, however, as found by Maynard (27), the timothy hay is much lower in calcium than is the hay found satisfactory in Wisconsin and Pennsylvania. Fifty samples, gathered at full bloom from good and poor soils, fertilized and unfertilized, ranged from 0.14 to 0.35 per cent calcium with a mean of 0.24 per cent. It is understandable that some of the timothy hay grown in New York State does not provide satisfactory nutrition for dairy cows even though the hay grown in Wisconsin or Pennsylvania may do so.

In general the calcium content of plants is lowest on acid soils. This has been shown by Zimmerley (28) who concludes that it is not due to an actual lack of the element in the soil but to an inability of the roots to absorb that present. Salgues (15) found somewhat more calcium in both legumes and grasses on calcareous than on siliceous soils. Marked differences in the calcium content of vegetables in accordance with the nature of the soil have been reported (21, 22). Contrary to the general observation with phosphorus, an increase in rainfall appears to cause a decrease in the calcium content of forage (11).

Whether or not the calcium content of crops can be increased by fertilizer treatment depends on whether the soil reaction and other conditions are favorable for calcium absorption. Zimmerley (28) found that heavy applications of superphosphate increased the calcium content of lettuce, beets, and carrots at different soil acidities. Working with a different soil, Bishop (21) found that superphosphate applications increased the phosphorus but not the calcium content of cabbage and lettuce. In fact the calcium content generally decreased. Similarly Elmendorf & Pierce (23) found that various fertilizer treatments which increased the phosphorus content of vegetables tended to cause a decrease in calcium rather than otherwise.

The indications that the calcium and phosphorus content may vary inversely under the influence of moisture and certain fertilizer applications are of special interest because of the intimate association of

these elements in animal metabolism and the importance of having
them present in a certain ratio to each other. In the areas of phosphorus
deficiency the calcium content of the forage is generally high. Such is
the case in Minnesota (11) and Texas (12). The calcium-phosphorus
ratio is sometimes as wide as ten to one. This may be a contributing
factor, along with the inadequate phosphorus, in the severe animal
troubles. In the low phosphorus area in Tennessee (4, 5), legumes
grow, providing a forage rich in calcium, though the lespedeza studied
contained 30 per cent more when grown in the phosphorus-rich Basin
area. This difference for calcium is small, however, compared to the
nearly three-fold difference in phosphorus content noted in the hay
in the two areas.

Previous discussion has indicated that on the acid, sandy soils of
Florida both calcium and phosphorus tend to be low in the crops. In
British Guiana (18), the natural, mixed pasture grass, containing the
very low figure of 0.01 per cent phosphorus on a dry basis was de-
ficient in calcium also, as indicated by a figure of 0.1 per cent. But
the introduced Wynne grass contained nearly 1 per cent calcium
although the phosphorus content was very low.

Iron.—There are large areas in Florida where a disease known as
"salt sick," characterized by anemia, has been known for over forty
years. In 1931 Becker, Neal & Shealey (29) reported recovery of
animals and restoration of hemoglobin after the administration of fer-
rous ammonium citrate which they recognized to contain a trace of
copper. Later Neal & Becker (30) made hemoglobin determinations
in thirty-nine herds and reported that the values were correlated with
the physical symptoms of "salt sick." Cures were obtained with fer-
rous ammonium citrate and copper sulfate. They felt that the iron
deficiency explanation was supported by their analyses of the forage
(31). Bryan & Becker (32) have described the soils on which cattle
develop "salt sick." The surface soil in healthy areas was found to
contain approximately ten times as much iron, twice as much copper,
and five times as much phosphorus and calcium as that in the sick
areas. The cattle develop trouble on soils with 0.036 per cent iron
and 3.85 p.p.m. copper. These data suggested that multiple defi-
ciencies may be involved in "salt sick" areas. Particularly because of
their later work which is referred to elsewhere in this review, the
Florida workers now believe that no single cause is responsible for
the cases of "salt sick" throughout the State and that other deficiencies
such as copper and cobalt may be more important than iron.

The investigations of Abbott and associates (33, 34, 35) on human anemia, however, make it clear that there are areas in Florida where the food crops are very low in iron because of soil conditions. In these same areas "salt sick" occurs. Abbott and co-workers found the highest incidence of anemia on poor soils, and that the vegetables grown on these soils were very low in iron. The iron content of turnip tops varied from 90 p.p.m. on a poor soil to 318 parts on rich soils. The daily iron intakes of representative families ranged from 1.2 to 16 mg. of iron per person. In a study of eight series of Florida soils, Rogers and co-workers (36) found the total iron content to be less than 0.14 per cent, with the exception of two series. In connection with this Florida evidence of iron deficiency in the human diet, it is interesting to note the reports from Mississippi (37), Georgia (38), and South Carolina (39) that the diets of many rural families are more often deficient in iron than any other mineral.

Archibald and associates (40) have studied a condition known as "neck ail" occurring in the Buzzards Bay area of Massachusetts. This trouble is an anemia accompanied by loss of appetite and emaciation. Iron ammonium citrate was curative. The forage was found to be 50 per cent lower in iron, and the soils one third lower in total iron, in the "sick" areas, as compared with the values for forage and soil in "healthy" areas. The available iron was 5 per cent of the total in both areas. In pot experiments with "sick" soils, iron fertilization increased the iron of the forage by 28 per cent. The authors feel that copper is ruled out as a cause of "neck ail."

Copper.—More than a decade before the role of copper in hemoglobin formation was discovered, investigators in Northern Europe were studying possible mineral deficiencies in the forage in areas where a wasting disease of cattle, called "lechsucht," was common. In 1933 Sjollema (41) established its cause as a copper deficiency by finding marked differences in the copper content of the forage in "healthy" and "sick" areas and by curing the trouble with copper therapy. Recently Sjollema (42) has shown that in affected cows and goats the copper content of the blood falls to one third of the normal concentration of 100 μg. per cent. The copper content of the liver, spleen, and hair was also decreased. It has also been established that a disease of lambs, referred to as enzootic ataxia or "swayback," and by other names, and characterized by nervous symptoms, is caused by a deficiency of copper in the ration of the pregnant ewe; it has been reviewed by Marston (43). The disease has been reported from Eng-

land, Sweden, Australia, and elsewhere. Bennetts & Hall (44) have reported preliminary results suggesting that a deficiency of copper may be concerned in a trouble known as "falling disease" and characterized by staggering, falling, and instantaneous death. It is apparent that these various troubles have certain symptoms in common and others which are not. Further study of the specific pathology of each is needed. A role of copper more general than its effect on hemoglobin formation is suggested.

It has been found that some of the areas of cobalt deficiency in Australia, discussed elsewhere in this review, are deficient in copper also and that both deficiencies contribute to the stock losses which occur among grazing animals. Becker and associates (45) have used copper salts successfully in treating several cases of anemia in cattle in Florida. These results, together with findings (36, 46) that certain soils in the state are very low in this element, suggest that a deficiency of copper may be a significant cause of the anemic troubles known as "salt sick," discussed elsewhere in this review. Rusoff (47), one of the Florida workers, has made an extensive study of the copper content of the tissues of newborn calves from normal and "salt sick" areas. No significant differences were observed. This thesis also contains data on many other trace elements together with over four hundred references.

Nicolaisen & Seelback (48) have shown that "lechsucht" can be prevented by copper fertilization. The influence of this fertilization in increasing the yield and absorbed copper of crops grown in the copper-deficient soil of South Australia has been shown by Riceman, Donald & Evans (49); and of West Australia by Teakle and co-workers (50). Miller & Mitchell (51) obtained an increase in the copper in lettuce but not in spinach, through fertilization with copper sulfate. Eisenmenger & Holland (52) have also reported an increase in the copper content of plants through fertilization.

Cobalt.—The history of the investigations of variously named troubles in animals now recognized to be caused by cobalt deficiency in the soil and herbage has been summarized by Marston (53). Recent reviews of various aspects of cobalt nutrition have been presented by Marston (43) and by Underwood (54).

The relationship between the cobalt content of the soil and forage and the state of health of cattle and sheep appears to be reasonably close. Soils containing less than 2.0 p.p.m. of cobalt commonly represent "sick" areas, while on soils having amounts in excess of this

figure, the animals are generally healthy. Wunsch (55) reports that the forage on thirty-nine "healthy" pastures averaged 0.106 p.p.m. of cobalt whereas figures of 0.07 p.p.m. and lower characterized "sick" areas. Several of the workers agree, however, that soil cobalt surveys have not been sufficiently extensive to make them an entirely reliable guide for differentiation between sound and abnormal areas.

Sheep suffering markedly from cobalt deficiency have been found to recover completely with daily intakes as small as 0.1 mg. Even more surprising is the response from soil applications. Bonner and others (56) have reported that an application of 28 oz. of cobaltous sulfate per acre increased the cobalt content of the pasturage from an unhealthy level of 0.06 p.p.m. to a healthy level of more than 0.1 p.p.m., a level which was maintained for at least a year. This rate of application, and even lesser ones, have been found to restore animals to health on "sick" areas. The marked increase in the cobalt content of the plant from these very small applications to the soil is particularly amazing because there is no convincing evidence that cobalt is an essential element for the growth of higher plants.

Bowstead & Sackville (57) have reported evidence of cobalt deficiency in western Canada. It is apparent that cobalt deficiency is at least one cause of some of the cases of "salt sick" in Florida. Neal & Ahmann (58) fed calves a ration based on hay from a suspected area. A failure in appetite and growth was noted, and the animals responded to cobalt administration. There was no relation between this condition and the hemoglobin level. The authors state that no cobalt was detected spectrographically in their basal ration, but they also refer to the studies at the Florida Station which failed to find cobalt spectrographically in the forage from either "sick" or "healthy" areas. The usefulness of spectrographic methods for the determination of small amounts of cobalt appears uncertain. Since the first report by Neal & Ahmann, further evidence for the successful treatment of "salt sick" by cobalt has been briefly noted by Becker et al. (45).

The only extensive data on the cobalt content of soils in the United States is that of Slater, Holmes & Byers (59). Their data for six different horizons and nine different soil types contain no values as high as 2 p.p.m. and thus are surprisingly low figures. Rogers and associates (36) could find no cobalt in Florida soils spectrographically. Data on the cobalt content of plants and foods produced in the United States have been published by McHargue (60) and by Ahmad & McCollum (61).

The specific role of cobalt in the various area troubles which respond to its administration has not yet been established. Several recent papers support the viewpoint that the element plays a definite role in hemopoiesis. Kleinberg and associates (62) rendered rabbits anemic by repeated bleedings or by benzol administration. Injections of 50 mg. of hydrated cobaltous nitrate daily resulted in rapid recovery in blood cell count. It was indicated that the cobalt produced this effect by stimulating the formation of erythrogenic precursors in the bone marrow. Polonovski & Breskas (63) have studied the hemopoietic action in anemia produced in growing rats. Frost, Elvehjem & Hart (64) reported that dogs made anemic by copper deficiency and then kept on a milk diet made an excellent response to iron and copper but that the hemoglobin reached a plateau at 10 to 12 gm. per 100 cc. blood. As little as 0.1 mg. of cobalt daily stimulated hemoglobin production in these dogs and allowed complete regeneration. Orten & Orten (65) have reported that cobaltous chloride given orally to rats which had been made anemic on a low protein diet resulted in a steady increase in hemoglobin concentration. Kato (65a) has reported cases where iron plus cobalt was more effective in treating anemia in children than iron alone.

While anemia commonly occurs in animals on cobalt-deficient areas and while there is considerable evidence for a direct relationship here, the physical symptoms have been produced experimentally without anemia (54). The apparent lack of relationship between the state of cobalt nutrition and hemoglobin level in the Florida studies has been referred to (58). The viewpoint has been expressed that the anemia commonly noted in cobalt-deficient animals may be secondary to the inanition which always results. As is discussed elsewhere in this review, in some of the areas under study there is evidence that a lack of copper is present also and may be responsible for the anemic symptoms.

It is apparent that the requirements of cobalt per unit of body weight are lower for cattle than for sheep. Proof that the element is necessary for other species has not been produced. Horses thrive in the same areas where cattle and sheep develop serious trouble (54). Attempts by Underwood & Elvehjem (66) to produce cobalt deficiency in rats by the use of a purified diet proved unsuccessful. Their carefully controlled work makes clear the problem of devising an experimental diet adequate in other respects which will not contain the very small amount of cobalt which may be required. Success in this

direction with any species would greatly aid in clearing up the specific role of cobalt. Another need, which is apparent from the previous discussion and from literature not cited, is a more precise analytical procedure for cobalt. This fact should be borne in mind in considering the quantitative data thus far published.

Other minerals.—The iodine problem is not considered in this review because it has been reviewed so frequently elsewhere. The recent book by McClendon (67) should be mentioned. It is an exhaustive compilation dealing with the distribution of iodine in soils, water, foods of animal and vegetable origin, and in the body. It is written primarily for the use of research workers. While it has been shown that there are soil types associated with manganese deficiency in plants there is no demonstrated relationship to animal nutrition. Many experiments, illustrated by the studies of Davidson & LeClerc (22), have shown that the crop content of other elements essential in animal nutrition, such as sodium, potassium, magnesium, and chlorine, can be influenced by soil factors, but they do not present any nutritional problem. Many recent studies have shown that there are soils where a lack of boron markedly interferes with plant growth and development, but studies by Hove and co-workers (68) failed to obtain any evidence that this element is needed in animal nutrition. A comprehensive review of magnesium in animal nutrition has recently been published by Duckworth (69). The Imperial Bureau of Soil Science (70) has just issued a very extensive review of the minor elements of the soil, containing over four hundred citations.

Toxic Constituents

The following discussion deals with elements or substances originating in the soil which have no known useful function in animal nutrition and which may occur in food and water in amounts which are harmful.

Arsenic and lead.—The extensive use of arsenicals, particularly lead arsenate, in insect pest control and in weed eradication has raised important questions regarding the fate of the arsenic and lead thus added to the soil and the possible influence on plants, particularly those used for food. Vandecaveye and co-workers (71) investigated the accumulation of these minerals as a cause of the loss of productivity in old orchard soils. They found that the poor condition of alfalfa and barley was correlated with the soluble arsenic in the soils. In pot tests with barley grown in soils from old orchards, the tops

contained 10 to 17 p.p.m. of arsenious oxide while the roots contained
788 to 1,640 p.p.m. The readily soluble lead in the soil was very small
and the barley tops contained only a trace, but the roots contained
662 to 756 p.p.m. This localization of lead in the roots has been ob-
served by others. Arsenic rather than lead was considered to be the
primary factor affecting plant growth. There is abundant evidence
that the contamination of soils by arsenic lowers the yields of various
crops. Apparently the extent of the effect depends upon the kind of
soil and kind of plant as well as the amount of arsenic present.

Keaton (72) reports that applications of lead carbonate in amounts
as high as 7,190 pounds per acre had no detrimental effect on barley
growth, due to the high fixing power of the soil used. He found an
accumulation of lead in the plants, however, principally in the roots,
which was proportional to the amount of lead added to the soil. For
example, the addition of 60 p.p.m. of lead nitrate resulted in 154 p.p.m.
in the roots and 0.77 p.p.m. in the tops, whereas the addition of
500 p.p.m. produced plants containing 617 p.p.m. and 2.6 p.p.m. in
the roots and tops respectively.

Williams & Whetstone (73) have published some important studies
of the arsenic content of various soils. From the analysis of some
sixty different kinds of soil believed free from added arsenic, from
different parts of the United States, they found that arsenic was always
present, ranging from 0.3 to nearly 40 p.p.m. Soils contaminated
with arsenicals contained many times as much of the element in the
"surface few inches," the maximum quantity found being 550 p.p.m.
Williams & Whetstone report data for the arsenic content of over one
hundred samples of vegetation growing on normal soils. Most of the
values were less than 1 p.p.m. Markedly higher values were found in
crops grown on soils contaminated with arsenicals. It seems apparent
that while the primary effect of excessive soil arsenic is upon plant
growth, the possibility of toxic foodstuffs from such soils cannot be
excluded. On the basis of preliminary evidence Hurd-Karrer (74)
has suggested that phosphate applications will reduce or prevent ar-
senic injury to plants where the type of soil is such as to permit the
phosphorus to remain available and that this effect might be of con-
siderable importance in soils contaminated with spray residues.

Grimmett and associates (75, 76, 77) have reported sickness and
death among animals grazing drained swamp land in the Waiotapu
Valley of New Zealand. The soil contained from 0.008 to 0.525 per
cent of arsenic and the water contained from a trace to 2.6 grains

of arsenic pentoxide per gallon. From experimental observations with animals, including analyses of organs for arsenic content, it was concluded that arsenic ingestion was responsible for both the acute and chronic cases, and that the cattle obtained the arsenic from the water, and from eating the mud along the drains in the absence of adequate herbage.

Fluorine.—While fluorine troubles in farm animals from the feeding of fluorine-containing phosphorus supplements have been known for a dozen years, the recognition has been much more recently made that these troubles are also endemic in areas where "mottled enamel" occurs in children. These area troubles and their soil relationships have been recently reviewed in an excellent manner by Peirce (78). He cites the literature regarding long-existing troubles in Northern Africa, Iceland, and the Argentine, as well as in the United States. It is stated that chemical investigations of the soils, vegetation, and bones of animals in the affected areas indicate a fluorine content as much as ten times that of similar materials from healthy areas. Peirce cites many cases of fluorine poisoning in animals grazing around factories which contaminate the soil or vegetation with fluorine wastes.

Commercial phosphates used as fertilizer contain 3 to 4 per cent of fluorine. In limited studies Hart and co-workers (79) concluded that this does not involve any toxic hazard in so far as increased fluorine content of the crops is concerned. They did find, however, that the fluorine content was significantly increased, and, more important, that the drainage waters from fertilized plots were high in the element. It appears from the work of Machle, Scott & Treon (80) that there may be no direct correlation between food fluorine and water fluorine, and that food is relatively unimportant compared to water as a source of fluorine intake. But this may not be the case in all areas. Further studies seem desirable to ascertain whether the continuous heavy phosphate manuring which is practiced for intensive crop production in certain areas may be adding fluorine to the water supplies and food of animals and man in amounts which may be potentially dangerous.

Within the past year some experimental evidence has appeared that dental caries in rats may be reduced by adding a limited amount of fluorine to the diet. Several papers have also reported observations indicating that there is less dental caries in children in "mottled enamel" areas and that there may be a physiologically normal fluorine intake which promotes resistance to caries. These papers have been

reviewed by Cox (81). In this connection a paper by Smith & Smith (82), the pioneer workers in the field of mottled enamel, is highly pertinent. They warn against any plan to build caries resistance by adding fluorides to food or water supplies.

Selenium.—The status of work on the selenium problem up to 1937 has been very comprehensively reviewed by Moxon (83). He has also covered some of the later literature in a bulletin which comprises a very complete report of the geological relationships of the occurrence of the element in soils (84).

Recent research emphasizes the widespread occurrence of selenium in soils and the need for further study of food crops in various areas. Williams, Lakin & Byers (85), of the U.S. Department of Agriculture, have issued during the past year a fourth report dealing with the occurrence of selenium in soils in the United States. Toxic areas have been located in several counties in Montana. Studies in other areas in the United States and its possessions are reported. Selenium was established as the cause of a 200-year-old disease in cattle and horses which occurs near Irapuato, Mexico. In addition to the examination of soils and vegetation collected in the area, vegetables from the public market were analyzed and found to contain from 0 to 70 p.p.m. of selenium. It is considered that the element is at least a contributing factor to the ill health of the people in the area. This bulletin by the Federal workers also reviews recent literature and topics related to the selenium problem. It deals with indicator plants as does also a publication by Beath and associates (86). Byers & Lakin (87) have reported on a reconnaissance examination revealing the existence of an enormous area of seleniferous soil in Canada. Robinson (88) has reported data indicating that selenium is probably a constituent of wheat wherever grown, reflecting its general occurrence in the soil.

Lakin and co-workers (89) have discussed the lack of quantitative correlation between the selenium content of the soil and the vegetation, explainable on the basis of variations in the form of the soil selenium, the character of the soil, and selective absorption by plants. Williams and co-workers (85) have reported that six different vegetables raised in an irrigated garden on highly seleniferous soil in North Dakota contained selenium below the limits normally considered toxic.

An interesting effect of sulfur and sulfate in selenized soils on the toxicity of wheat (90) and millet (91) grown in pot experiments has been reported. These observations were not confirmed in field tests (92, 93), presumably because of the different conditions present such

as type of selenium compounds, moisture relations, and other soil factors. Moxon and associates (94, 95) have reported studies showing the effectiveness of arsenic salts added to a diet of toxic wheat in preventing selenium poisoning in rats. No explanation of the antagonistic action is available. Smith (96) has reported from experiments with rats that the toxicity of naturally occurring, food selenium is largely determined by dietary factors. A high protein, low carbohydrate diet tends to protect against injury. Painter & Franke (97) have reported on the metabolic relationships of selenium, sulfur, and nitrogen deposition in cereals. Williams and co-workers (85) have reviewed recent studies with various species regarding the pathology of selenium poisoning.

Molybdenum.—A trouble referred to as "teartness," known for over a hundred years to be definitely associated with certain pasture areas in England and not with others, has been established as a molybdenum toxicity. The trouble affects ruminants, particularly calves and cows in milk. The physical symptoms are extreme diarrhea with consequent loss in weight and milk yield. In 1938 Ferguson and associates (98) reported studies of the herbage in the unhealthy areas, showing it to be high in molybdenum compared to that of normal areas. They fed small amounts of soluble molybdates to cows and produced the same symptoms noted in the animals grazing forage high in the element. A later publication by Ferguson and associates (99) gives a much more complete report. Herbage from healthy areas averaged less than 0.0005 per cent molybdenum on a dry matter basis, while the content in samples from "teart" areas ranged from 0.003 to 0.008 per cent. It was shown that a considerable part of the molybdenum fed was absorbed and excreted in the urine but no clue was obtained as to its specific action. The feeding of 2 gm. of copper sulfate per day to cows generally cured the diarrhea. This bulletin also discusses the uptake of molybdenum by various crops, the relation of the nature of the soil, the effect of fertilizers, and various management practices.

Nitrites.—In 1937 Newsom and co-workers (100) reviewed a series of reported cases of acute symptoms and death in cattle following the ingestion of oat hay or straw and referred to as "oat hay poisoning." These cases, which had occurred in their own state (Colorado) and in Wyoming, dated back to 1923. The physical symptoms noted were trembling, staggering gait, rapid respiration, prostration. In investigating a current case the workers obtained evidence from

feeding experiments that the oat forage was definitely responsible. Further evidence from the same laboratory was published a year later by Thorp (101).

Detailed studies of cases in Wyoming have been reported by Bradley and co-workers (102). On one ranch 45 out of 110 head of cattle died within two days following the beginning of the feeding of a particular lot of oat hay. Among the post-mortem findings recorded was the significant one that most of the hemoglobin had been converted to methemoglobin, explaining the physical symptoms noted. Chemical analyses of fifteen samples of oat hay and straw fed where deaths had occurred revealed a potassium nitrate content of 2.2 to 7.3 per cent, whereas other oat hay contained only a trace. A water extract of toxic hay, containing 128 gm. of potassium nitrate produced the typical symptoms in a calf, while an extract from a similar amount of hay, but with most of the potassium nitrate removed, caused no evident trouble.

Since nitrite, rather than nitrate, was known to form methemoglobin and since there was no nitrite present in the hay, Bradley and co-workers concluded that the nitrate must have been converted into nitrite in the digestive tract. They found large amounts of nitrite in the urine and bile of affected animals. Support of their conclusion is to be found in earlier studies by Seekles & Sjollema (103) who introduced potassium nitrate into the rumen of cattle and got symptoms of nitrite poisoning as indicated by the formation of methemoglobin. They obtained evidence that about 10 per cent of the nitrate injected was changed to nitrite in the rumen.

The earlier literature also contains a most interesting and unusual case of poisoning from forage high in nitrate studied by Remington & Quin (104) in South Africa. For some time it had been known that a characteristic disease of sheep was caused by grazing on certain species of *Trebulus* plants. The investigators showed that the formation of methemoglobin was a typical finding, that it could be produced by the plant juice or aqueous extracts of the plant, and that nitrite was the causative agent. They found large amounts of nitrate in the plants but generally only traces of nitrite. From various studies they drew the conclusion that under certain field conditions nitrite was produced from nitrate in the plant under the influence of an enzymic oxidation-reduction system.

Limited observations by the Wyoming workers suggest that horses and sheep, as well as cattle, may occasionally suffer from "oat hay"

poisoning. Other plants, such as certain weeds, wheat, barley, and cane sorghum, sometimes contain enough nitrate to cause the characteristic trouble. It is apparent that the troubles are limited to certain areas, with some evidence that they occur year after year in these areas. The limited soil analyses and pot experiments which have been carried out thus far have produced little evidence regarding the soil factors concerned. Because of his recognition that nitrate may be changed into nitrite in the body, Sjollema (105) early studied the nitrate content of pasture grass as influenced by various factors. He found no correlation with type of soil or method of manuring. The ranges studied were 0.5 to 2 per cent in contrast to the higher values found by Bradley and co-workers in the toxic hay.

It appears that "oat hay poisoning" is of most concern in the case of ruminants because of the special opportunity for chemical changes provided by their digestive tracts. No cases have been reported in omnivora. There appears to be reason, on the basis of the present evidence, for concern regarding the possibility that nitrate fertilization may produce crops which are toxic.

LITERATURE CITED

1. AUCHTER, E. C., *Science,* **89**, 421 (1939)
2. BEESON, K. C., *U.S. Dept. Agr. Misc. Pub.,* No. 369 (In press)
3. THEILER, A., GREEN, H. H., AND DU TOIT, P. J., *J. Dept. Agr. Union S. Africa,* **8**, 460 (1924)
4. WEATHERS, E. K., *Tenn. Agr. Expt. Sta. Bull.,* No. 166 (1938)
5. ANONYMOUS, *Tenn. Agr. Expt. Sta. Popular Bull.,* No. 1 (1940)
6. ANONYMOUS, *U.S. Dept. Agr., Yearbook,* 1063, 1068 (1938)
7. WILLIAMS, D. E., MACLEOD, F. L., AND MORRELL, E., *J. Nutrition,* **19**, 251; **20**, 391 (1940)
8. BECKER, R. B., NEAL, W. M., AND SHEALY, A. L., *Florida Agr. Expt. Sta. Bull.,* No. 264 (1933)
9. BRYAN, O. C., AND NEAL, W. M., *J. Agr. Research,* **52**, 459 (1936)
10. ROGERS, L. H., GALL, O. E., GADDUM, L. W., AND BARNETTE, R. M., *Florida Agr. Expt. Sta. Tech. Bull.,* No. 341 (1939)
11. ECKLES, C. H., BECKER, R. B., AND PALMER, L. S., *Minn. Agr. Expt. Sta. Bull.,* No. 229 (1926)
12. SCHMIDT, H., *J. Am. Vet. Med. Assoc.,* **96**, 441 (1940)
13. MCCREARY, O. C., *Wyoming Agr. Expt. Sta. Bull.,* No. 233 (1939)
14. DANIEL, H. A., AND HARPER, H. J., *J. Am. Soc. Agron.,* **27**, 644 (1935)
15. SALGUES, R., *Ann. agron.,* **8**, 325 (1938)
16. LUSH, R. H., *Proc. Assoc. Southern Agr. Workers,* 75 (1939)
17. MCGEORGE, W. T., *Ariz. Agr. Expt. Sta. Tech. Bull.,* No. 82 (1939)

18. DUTHIE, D. W., *Agr. J. Brit. Guiana,* **10,** 194 (1939)

19. LIEBENGOOD, H., *Vet. Med.,* **34,** 490 (1939)

20. FORBES, E. B., AND JOHNSON, S. R., *Penna. Agr. Expt. Sta. Bull.,* No. 371 (1939)

21. BISHOP, E. R., *J. Nutrition,* **8,** 239 (1934)

22. DAVIDSON, J., AND LECLERC, J. A., *J. Nutrition,* **11,** 55 (1936)

23. ELMENDORF, E., AND PIERCE, H. B., *J. Nutrition,* **20,** 243 (1940)

24. BECKER, R. B., NEAL, W. M., AND SHEALY, A. L., *Florida Agr. Expt. Sta. Bull.,* No. 262 (1933)

25. HART, E. B., HADLEY, F. B., AND HUMPHREY, G. C., *Wisconsin Agr. Expt. Sta. Research Bull.,* No. 112 (1932)

26. BECHDEL, S. I., WILLIAMS, P. S., SHINGLEY, J. F., AND BORLAND, A. A., *Penna. Agr. Expt. Sta. Bull.,* No. 395 (1940)

27. MAYNARD, L. A., *J. Am. Soc. Agron.,* **29,** 504 (1937)

28. ZIMMERLEY, H. H., *Virginia Truck Expt. Sta. Bull.,* No. 73 (1930)

29. BECKER, R. B., NEAL, W. M., AND SHEALY, A. L., *Florida Agr. Expt. Sta. Bull.,* No. 231 (1931)

30. NEAL, W. M., AND BECKER, R. B., *J. Agr. Research,* **46,** 557 (1933)

31. NEAL, W. M., AND BECKER, R. B., *J. Agr. Research,* **47,** 249 (1933)

32. BRYAN, O. C., AND BECKER, R. B., *J. Am. Soc. Agron.,* **27,** 120 (1935)

33. ABBOTT, O. D., *Florida Agr. Expt. Sta. Ann. Rept.,* 71 (1936)

34. ABBOTT, O. D., AND AHMANN, C. F., *Florida Agr. Expt. Sta. Bull.,* No. 328 (1938)

35. ABBOTT, O. D., AND AHMANN, C. F., *J. Nutrition,* **19,** 9 (1940)

36. ROGERS, L. H., GALL, O. E., GADDUM, L. W., AND BARNETTE, R. M., *Florida Agr. Expt. Sta. Tech. Bull.,* No. 341 (1939)

37. DICKINS, D., *Miss. Agr. Expt. Sta. Bull.,* No. 245 (1927)

38. MATTHEWS, S., *Georgia Agr. Expt. Sta. Bull.,* No. 159 (1929)

39. MOSER, A. M., *S. Carolina Agr. Expt. Sta. Bull.,* No. 300 (1935)

40. ARCHIBALD, J. G., KUCINSKI, K. J., BROOKE, R. O., AND FREEMAN, S. L., *J. Dairy Sci.,* **21,** 59 (1938)

41. SJOLLEMA, B., *Biochem. Z.,* **267,** 151 (1933)

42. SJOLLEMA, B., *Biochem. Z.,* **295,** 372 (1938)

43. MARSTON, H. R., *Ann. Rev. Biochem.,* **8,** 573 (1939)

44. BENNETTS, H. W., AND HALL, H. T. B., *J. Dept. Agr. W. Australia,* **16,** 156 (1939)

45. BECKER, R. B., NEAL, W. M., ARNOLD, P. T. D., AND RUSOFF, L. L., *Florida Agr. Expt. Sta. Ann. Rept.,* 68 (1939)

46. PEECH, M., *Florida Agr. Expt. Sta. Tech. Bull.,* No. 340 (1939)

47. RUSOFF, L. L., *Thesis* (University of Minnesota Graduate School, 1940)

48. NICOLAISEN, W., AND SEELBACK, W., *Forschungsdienst,* **5,** 383 (1938)

49. RICEMAN, D. S., DONALD, C. M., AND EVANS, S. T., *Australia Council Sci. Ind. Research Pamphlets,* No. 96 (1940)

50. TEAKLE, L. J. H., TURTON, A. G., AND THOSSELL, G. L., *J. Dept. Agr. W. Australia,* **17,** 161 (1940)

51. MILLER, L., AND MITCHELL, H. S., *J. Am. Dietet. Assoc.,* **7,** 252 (1931–32)

52. EISENMENGER, W. S., AND HOLLAND, E. B., *Mass. Agr. Expt. Sta. Ann. Rept. 1936,* Bull. 339, 11 (1937)

53. MARSTON, H. R., *Australia Council Sci. Ind. Research Bull.*, **113**, 14 (1938)
54. UNDERWOOD, E. J., *Nutrition Abstracts & Revs.*, **9**, 520 (1940)
55. WUNSCH, D. S., *Chemistry & Industry*, 531 (1939)
56. BONNER, W. G., McNAUGHT, K. J., AND PAUL, G. W., *New Zealand J. Agr.*, **58**, 493 (1939)
57. BOWSTEAD, J. E., AND SACKVILLE, J. P., *Can. J. Research, D*, 17, 15 (1939)
58. NEAL, W. M., AND AHMANN, C. F., *J. Dairy Sci.*, **20**, 741 (1937)
59. SLATER, C. S., HOLMES, R. S., AND BYERS, H. G., *U.S. Dept. Agr. Tech. Bull.*, No. 552 (1937)
60. McHARGUE, J. S., *J. Agr. Research*, **30**, 193 (1925) ; *Ind. Eng. Chem.*, **19**, 274 (1927)
61. AHMAD, B., AND McCOLLUM, E. V., *Am. J. Hyg.*, **29**, *Sec. A*, 24 (1939)
62. KLEINBERG, W., GORDON, A. S., AND CHARIPPER, H. A., *Proc. Soc. Exptl. Biol. Med.*, **42**, 119 (1939)
63. POLONOVSKI, M., AND BRESKAS, S., *Compt. rend. soc. biol.*, **130**, 1588 (1939)
64. FROST, D. V., ELVEHJEM, C. A., AND HART, E. B., *J. Nutrition*, **19**, 311 (1940)
65. ORTEN, J. M., AND ORTEN, A. U. (Paper presented before Division of Biological Chemistry, American Chemical Society, Detroit, 1940)
65a. KATO, K., *Am. J. Pediat.*, **11**, 385 (1937)
66. UNDERWOOD, E. J., AND ELVEHJEM, C. A., *J. Biol. Chem.*, **124**, 419 (1938)
67. McCLENDON, J. F., *Iodine and the Incidence of Goiter* (Oxford University Press, London, 1939)
68. HOVE, E., ELVEHJEM, C. A., AND HART, E. B., *Am. J. Physiol.*, **127**, 689 (1939)
69. DUCKWORTH, J., *Nutrition Abstracts & Revs.*, **8**, 841 (1939)
70. IMPERIAL BUREAU OF SOIL SCIENCE, *Tech. Commun.*, No. 39 (1940)
71. VANDECAVEYE, S. C., HORNER, G. M., AND KEATON, C. M., *Soil Sci.*, **42**, 203 (1936)
72. KEATON, C. M., *Soil Sci.*, **43**, 401 (1937)
73. WILLIAMS, K. T., AND WHETSTONE, R. R., *U.S. Dept. Agr. Tech. Bull.*, No. 732 (1940)
74. HURD-KARRER, A. M., *J. Wash. Acad. Sci.*, **26**, 180 (1936)
75. GRIMMETT, R. E. R., AND McINTOSH, I. G., *New Zealand J. Sci. Tech.*, **21A**, 137 (1939)
76. FITCH, L. W. N., GRIMMETT, R. E. R., AND WALL, E. M., *New Zealand J. Sci. Tech.*, **21A**, 146 (1939)
77. GRIMMETT, R. E. R., McINTOSH, I. G., WALL, E. M., AND JONES, G. B., *New Zealand J. Sci. Tech.*, **21A**, 150 (1939)
78. PEIRCE, A. W., *Nutrition Abstracts & Revs.*, **9**, 253 (1939)
79. HART, E. B., PHILLIPS, P. H., AND BOHSTEDT, G., *Am. J. Pub. Health*, **24**, 936 (1934)
80. MACHLE, W., SCOTT, E. W., AND TREON, J., *Am. J. Hyg.*, **29**, 139 (1939)
81. COX, G. J., *J. Am. Dental Assoc.*, **27**, 1107 (1940)
82. SMITH, M. C., AND SMITH, H. V., *Am. J. Pub. Health*, **30**, 1050 (1940)
83. MOXON, A. L., *S. Dakota Agr. Expt. Sta. Bull.*, No. 311 (1937)

84. Moxon, A. L., Olson, O. E., and Searight, W. V., *S. Dakota Agr. Expt. Sta. Tech. Bull.,* No. 2 (1939)

85. Williams, K. T., Lakin, H. W., and Byers, H. G., *U.S. Dept. Agr. Tech. Bull.,* No. 702 (1940)

86. Beath, O. A., Gilbert, C. S., and Eppsom, H. F., *Am. J. Botany,* 27, 564 (1940)

87. Byers, H. G., and Lakin, H. W., *Can. J. Research, B,* 17, 364 (1939)

88. Robinson, W. O., *Ind. Eng. Chem., Ind. Ed.,* 28, 736 (1936)

89. Lakin, H. W., Williams, K. T., and Byers, H. G., *Ind. Eng. Chem., Ind. Ed.,* 30, 599 (1938)

90. Hurd-Karrer, A. M., *Am. J. Botany,* 25, 666 (1938)

91. Gile, P. L., Lakin, H. W., and Byers, H. G., *J. Agr. Research,* 57, 1 (1938)

92. Knight, S. H., and Beath, O. A., *Wyoming Agr. Expt. Sta. Bull.,* No. 221 (1937)

93. Franke, K. W., and Painter, E. P., *Ind. Eng. Chem., Ind. Ed.,* 29, 591 (1937)

94. Moxon, A. L., and DuBois, K. P., *J. Nutrition,* 18, 447 (1939)

95. DuBois, K. P., Moxon, A. L., and Olson, O. E., *J. Nutrition,* 19, 477 (1940)

96. Smith, M. I., *U.S. Pub. Health Repts.,* 54, 1441 (1939)

97. Painter, E. P., and Franke, K. W., *Am. J. Botany,* 27, 336 (1940)

98. Ferguson, W. S., Lewis, A. H., and Watson, S. J., *Nature,* 141, 553 (1938)

99. Ferguson, W. S., Lewis, A. H., and Watson, S. J., *Jealott's Hill Research Sta. Bull.,* 1 (1940)

100. Newsom, I. E., Stout, E. N., Thorp, Jr., F., Barber, C. W., and Groth, A. H., *J. Am. Vet. Med. Assoc.,* 43, 66 (1937)

101. Thorp, Jr., F., *J. Am. Vet. Med. Assoc.,* 45, 159 (1938)

102. Bradley, W. B., Eppsom, H. F., and Beath, O. A., *Wyoming Agr. Expt. Sta. Bull.,* No. 241 (1940)

103. Seekles, L., and Sjollema, B., *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.,* 2, 266 (1932)

104. Remington, C., and Quin, J. I., *Onderstepoort J. Vet. Sci. Animal Ind.,* 1, 469 (1933)

105. Sjollema, B., *Tierernähr.,* 2, 505 (1930–31); *Biedermanns Zentr. B. Tierernähr.,* 3, 507 (1931–32)

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MINERAL NUTRITION OF PLANTS

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In discussing the literature on the mineral nutrition of plants, the material is divided into two parts, (*a*) the absorption and accumulation of salts, and (*b*) the effects of the various elements on plant growth and metabolism.

Since the review last year, several papers presenting important work on the absorption and accumulation of salts by plants have appeared. Hoagland & Broyer have reported concerning the relation of the hydrogen-ion activity of the culture medium to salt accumulation. The particular aspects investigated were:

(a) the relation of the metabolic activities of root cells to changes of hydrogen-ion activity produced in the external medium as a result of selective ion absorption under the influence of metabolism, (b) relations of cation and anion accumulation to the metabolism and buffer systems of root cells, (c) effects of hydrogen-ion activity of culture solution on the accumulation of certain ions by the roots, with special reference to mechanisms of salt accumulation.

The observations made on changes of pH in the culture medium caused by excised roots were particularly valuable because of the promptness with which the initial observations were made and the frequency of subsequent observations. Thus with material from plants that were designated as "low salt" plants, there was a sharp drop within three hours from the initial pH value of about 5 to about pH 4 and a subsequent rise within twenty-one hours to a pH value of nearly 7. With roots of plants from cultures in which a higher salt concentration was maintained, there was an increase in pH value to about 7 within the first three to four hours. The pH values of both solutions were practically the same at the end of twenty-one hours.

A study of the effect of carbohydrate supply on pH was also included. Three sets of excised roots were used: those depleted of sugar; those depleted of sugar, to which glucose was administered; and normal roots. With sugar-depleted roots there was a slight initial drop in the pH of the culture solution to a little below 5, and then a steady rise to pH 7. With roots depleted of sugar, to which glucose had been administered, and with normal roots there was a drop in pH values for the first four hours and then a rise. Curves represent-

ing the results obtained for these two cases were nearly parallel. The curve for the sugar-depleted roots plus glucose lay about 0.3 pH units below that for the control. It reached a minimum of about pH 3.5 after four hours and rose to a pH value of about 5.7 after twenty-four hours. The curve for the sugar-depleted roots appeared to flatten out while the slopes of the other two curves were greatest at the final readings. The following quotation is of worth to workers in this field:

The general conclusion that may be drawn from these experiments is that the observed drift in the reaction of the culture solutions is not to be ascribed merely to the physical and chemical properties of different ions in relation to cell permeability, nor to ion exchanges such as may occur in non-living colloidal systems, but that it is also the result of the metabolic activities of the root which, regulated by oxygen, temperature, and initial salt and carbohydrate content, determine the differential rates of ion accumulation, and the consequent effects on the reaction of the external solution. A closely integrated system is envisaged with a large array of variables. Varying conditions of temperature, aeration, and pretreatment of tissues, governing the metabolic activities of root cells could lead to apparently divergent conclusions concerning the selective absorption of ions or pH changes.

In order to determine the effect produced by various types of salts on the buffer systems of roots, titration curves of composite samples of expressed sap were made. The sap was obtained from excised barley roots which had been exposed to aerated single salt solutions of potassium bicarbonate, potassium bromide, or calcium bromide. Although the entrance of potassium into the cells from a solution of potassium bicarbonate means a large accretion of free base, no increase in pH value of the expressed sap was found. Their titration curves on the acid side, however, indicated a considerable increase in the total organic acid content. Bromide was readily absorbed from solutions containing calcium bromide, but there was little if any absorption of calcium. As a result, a marked decrease in total organic acid in the sap occurred. From solutions of potassium bromide, both ions were absorbed in approximately equivalent amounts and little change in the total organic acid content was found.

The effects of the hydrogen-ion activity of the culture medium on salt accumulation by excised barley roots were also investigated. The accumulation of potassium, bromide, and nitrate ions from buffered phosphate solutions (initial pH range from 3.4 to 8.7, final range from 3.9 to 7.6) which were vigorously aerated, was not profoundly affected by the pH values of the solutions, although the accumulation of both cations and anions was somewhat greater for solutions

with initial pH values larger than 6. Marked accumulation of potassium and bromide ions—that is, absorption against a concentration gradient—took place at every pH value studied. There was a slight indication of an optimum range. An increase in the electrical conductivity of the expressed sap paralleled the increase in potassium concentration. In response to the suggestion that an adequate test of the [K] [OH] gradient hypothesis might require a study of potassium absorption in very dilute solutions, experiments were carried out in unbuffered solutions. In the first tests large volumes of 0.0001 M potassium bromide solution were used. The acidities of the solutions were adjusted with sulfuric acid and sodium hydroxide, and although the pH values could not be held entirely constant, two markedly different ranges were maintained. "Although the early rate of accumulation of K was somewhat greater from a solution of the pH ranges 6.6 to 7.4 than from one of pH 4.1 to 4.5, there appeared no difference in rates commensurate with the difference between the solutions in the [K] [OH] products, or H ion activities." In another test use was made of the carbonate–bicarbonate–carbon dioxide system for the maintenance of various pH values. The pH values of the solutions before the experiment ranged from 4.8 to 8.2 and at the conclusion of the experiment from 4.6 to 8.2. Here again, no relationship indicative of control by gradients of hydrogen ion-activity betwen sap and external medium, or thermodynamic potentials of [K] [OH] were observed.

Two papers, one by Steward & Preston, and the other by Steward, Stout & Preston, on salt accumulation and metabolic relations in potato discs, present much data of value concerning the uptake and accumulation of salts by plants. At oxygen tensions such that respiration was not limited by oxygen, the respiratory rate of the discs was increased by potassium bromide, potassium chloride, and potassium nitrate and decreased by calcium bromide and calcium chloride, as compared with distilled water. The effects of inorganic ions on respiration were found to parallel their effect on protein synthesis. Both the stable amide and amino acid fractions were found to be sources of nitrogen for protein formation in the dormant potato tuber. The bulk of the nitrogen converted to protein appeared to be from amino acids other than asparagine. Potassium salts appeared to increase and calcium salts to decrease the relative utilization of these amino acids. The absorption of the salts, potassium nitrate and potassium bromide, in which the uptake of the anion exceeded that of the cation, caused an increase in

the bicarbonate concentration and an alkalinity of the external solution. "In aerated solutions, potassium salts stimulated and calcium salts depressed water absorption in a manner not wholly explicable by osmotic phenomena." The conclusion was that sugar concentration does not regulate respiration rate since all treatments caused an increase in sugar concentration relative to that in the initial tissue, and salt and oxygen treatments which stimulated respiration produced a low residual sugar concentration. "At a low oxygen concentration (solution in equilibrium with 3.80 per cent O_2) at which accumulation of bromide was depressed, synthesis of protein from amino acid was also limited by oxygen lack." An approximate linear relationship was found between the amount of protein synthesized and the amount of carbon dioxide respired. The conclusion was reached that approximately one third of the respiration of discs in aerated distilled water occurs independently of nitrogen metabolism. This component of total respiration was apparently not affected by salts or oxygen concentration. Two thirds of the respiration of discs in distilled water appeared to arise from a component of respiration which is linked to protein synthesis from amino acids. This respiration was accelerated by favorable oxygen supply and by potassium and nitrate ions. It was depressed by low oxygen concentration and calcium ions. The characteristic effects of ions on respiration and synthesis were found to be exerted at oxygen tensions at which their absorption occurred, and the reactions of the ions observed were considered therefore to constitute an integral part of the machinery of salt absorption.

The absorption of ions has been correlated with the respiration of the potato discs. In order to thoroughly understand the mechanism of salt absorption and accumulation it is necessary to understand the energy changes taking place. For this reason an attempt has been made to correlate the energy relations with the chemical processes occurring. The balance sheet of metabolites presented in this paper showed that significant amounts of carbon (dry weight, carbohydrate) and calorific values were not accounted for when the tissue had respired in aerated water or potassium bromide solution until account was taken of organic substances removed from the surface of the discs by the drying papers used to remove the water. These losses were considerable at high oxygen tensions but were insignificant at low oxygen tensions and in the presence of calcium salts.

The surface film removed was formed at the expense of the starch fraction and consisted of a complex rich in uronic acid. If the uronic acid removed from the

discs by blotting is expressed as pectin the concomitant loss of carbon can be accounted for and also a large part of the loss of dry weight. . . . . Volatile organic compounds are produced by potato tissue under the conditions of these experiments only in small amount. Such compounds appear in the flowing gas stream and small amounts are evolved when the tissue is dried.

The author concluded that

not one, but the summation of *all* the metabolic and biochemical processes described—processes dependent upon oxygen—constitutes that "dynamic machinery" which is essential for salt accumulation.

This agrees with the statement of Hoagland & Broyer already quoted. The authors then proceed with the following statement:

The problem raised, however, is not only how these processes in living cells are directed to the accumulation of salts but *why they constitute such an apparently wasteful method of applying metabolic energy to the performance of useful work.*

This seems to preclude the assumption that salt accumulation may be made possible because of peculiar dynamic conditions caused when certain metabolic reactions take place, and that the amount of energy may be an apparent rather than a real measure of energy necessary for salt accumulation.

In the first of a series of papers (the others have not yet reached the reviewer), Stiles & Skelding reported the results of the absorption of potassium salts by storage tissue, carrot roots. The absorption of both ions of the potassium salts—chloride, bromide, nitrate, dihydrogen phosphate, and sulfate—were followed over a period of four to five days, by determining the decrease of salt concentration in the external solution. The absorption of both ions of all salts excepting the sulfate proceeded rapidly. It was found that the absorption of potassium ion was sometimes preceded by exosmosis from the tissues which was most obvious in the lowest concentrations. In higher concentrations, the absorption was usually greater than exosmosis, and the exosmosis was recognized only as a depression in the rate of absorption. Apparently the exosmosis was little affected, if at all, by the presence of the salt in the external solution. Exosmosis was, however, found to be much greater in a potassium dihydrogen phosphate solution than in any of the other salt solutions used. It was uncertain whether this was due to some difference in the particular lot of tissue examined or to a specific action of the salt, such as the free hydrogen ion. Absorption at first (two to three days) was found to be rapid.

After this there was a gradual decline with a subsequent increase in rate. The more rapid rate of absorption sometimes continued until the end of the experiment (usually four days) by which time practically the whole of the potassium ion had been absorbed from the solution. The explanations offered for the two-phase absorption were that it was

due to either an increase in permeability of cell membranes, as a result of exposure to a solution of the salt employed, or to absorption being of two kinds as hypothesized by Steward. . . . . The two ions of a salt are absorbed to unequal extents, but as such unequal absorption involves exosmosis of ions from tissue, the degree of unequal absorption depends on the extent to which exosmosis of ions is possible. Hence the departure from equality in the rate of absorption of kations and anions is more marked with dilute than with stronger solutions, since the concentration of ions available for exosmosis will be relatively less in relation to the quantities of ions absorbed the higher the concentration of the external solution.

Overstreet & Broyer reported studies on ionic interchange in which radioactive isotopes of potassium were employed. They set up experiments to observe the trends of exchange processes, basing their work on the supposition that

many if not all phase boundaries in the plant are permeable to cations in both directions, although the permeability to anion in one direction may be much greater than in the opposite direction . . . . [and] the trend toward isotopic equilibrium between culture medium and the plant root must involve a process of ionic exchange between isotopes.

The experimental technique took into consideration two major factors governing the entry of radioactive isotopes into the plant: first, the dependence on the inward movement of the particular ion species to which the isotope belongs; and second, the dependence of the influx on the distribution of the isotope in the various phases of the system. The first work was carried out with roots of "low salt" plants, plants showing the first symptoms of starvation in the shoots but with apparently healthy roots. There was no significant difference between the fractional uptake of radioactive and nonradioactive isotopes and, in plants with much capacity for the accumulation of all kinds of potassium, no trend toward isotopic equilibrium. Experiments were then conducted with roots of "high salt" plants, plants grown in complete nutrient solutions which had been renewed three times a week. When transferred to dilute potassium chloride containing radioactive potassium, these roots showed a decrease in total potassium content,

but at the same time absorbed radioactive potassium. A continuous exchange of radioactive potassium for nonradioactive potassium of the root throughout the nine-hour absorption period was observed. Experiments at low temperatures indicated that isotopic equilibrium may never be reached during the life period of the tissues. At 0° C. barley plants accumulated potassium from potassium chloride only in very small amounts, if at all. On the other hand, Broyer & Overstreet observed little effect of temperature on the exchange of radioactive potassium between roots and culture medium. At low temperatures only a certain part of the root appeared to be capable of attaining equilibrium with the outside medium within a moderate length of time. It was concluded that this part may represent the colloidal phases of the protoplasm and cell wall which are capable of rapid ionic exchange with the outside solution. The results indicated that only about 10 per cent of the total potassium in the roots of "low salt" plants is exchangeable at 0° C. Contrary to expectation, roots cultured at 0.5 C°. did not continue to accumulate radioactive potassium at an initially established rate. There was a decrease in rate to a low value in from three to five hours. They consider this suggestive of an

approach toward a steady state characteristic of the treatment, in which the net rates of the inward and outward movement are essentially equal . . . . [and] an inward movement initially involving primarily adsorption exchange of potassium isotopes.

Anaerobic conditions markedly decreased the net inward movement of the solute. An initial inward movement of the solute under anaerobic conditions was explained on the assumption of residual oxidative metabolism and possibly of certain instantaneous surface absorption phenomena. Later, under more strictly anaerobic conditions, further absorption was inhibited and the tissue failed to retain the small amount of radioactive potassium absorbed before anaerobiosis became completely effective.

In order to study the efflux of radioactive potassium, plants were exposed to solutions containing radioactive potassium previous to immersion in media devoid of this isotope. Roots from such plants retained cations nearly completely when placed in distilled water. When placed in potassium chloride solution, a net outward movement was observed. After a rapid initial decrease, the rate of this outward movement approached a small constant value. This outward movement was not affected by temperature. Less outward movement of

previously absorbed radioactive potassium was observed under aerobic than under anaerobic conditions. Broyer & Overstreet suggested that this was not caused entirely by reabsorption under more favorable conditions, but possibly by movement from external surfaces into protoplasm, vacuoles, and inner tissues, which removed from the field of exchange a large portion of the easily exchangeable potassium.

Schlenker reported an ingenious experiment devised to ascertain whether cations and anions adsorbed on permutite or on aniline black, are available to plants in an aqueous medium. Potassium, calcium, and magnesium permutite were prepared by leaching "Decalso" (an artificial zeolite) with the chlorides of the cations and washing with alcohol to remove the excess salts. The anion complexes were prepared by leaching three separate portions of "Demineralite" with nitric, phosphoric, and sulfuric acids, respectively. Potassium, calcium, and magnesium displaced sodium in the "Decalso." Acids, as such, were absorbed on the "Demineralite" (present trade name, "De-Acidite"). The complexes were mixed to simulate solution formulae used for growing plants, and a liter of water was added to each culture. The suspensions in which the plants were grown were aerated intermittently. Satisfactory results were obtained by the use of such culture media, and, although the data were too few for definite conclusions to be drawn, growth appeared to be better than in the corresponding solution cultures. Certain advantages of this method over the ordinary solution culture were suggested. In deficiency studies, a single ion, especially in the case of cations, may be added. In the case of anions, the accompanying hydrogen ion does not give low pH values; in fact, the pH values observed at the end of nineteen days showed remarkably little change from those at the beginning of the experiment. The total amount of nutrient for the whole growth period may be added at one time.

The Essential Elements

Conflicting data on the effects of various essential elements, potassium in particular, have appeared for years in plant nutrition literature. Reasons for such contradictory evidence were noted by Wall (1). The results reported by him in two subsequent papers and those in papers by Gauch, and by Richards & Shih do much to explain such differences. Undoubtedly there are great differences in the responses of different kinds of plants, but the effects of age, type of solution, and

other environmental factors should receive greater attention. Too little consideration is also given the fact that we may be observing indirect effects and, because of this, neglecting the precautions necessary for the demonstration of the specific effects we are seeking.

Boron.—In work which yielded evidence of interrelation in the effects of boron and indoleacetic acid on plant growth, Eaton has instituted a new and promising method of attack on the function of this element. Indoleacetic acid appeared to replace boron to some extent in its effect on growth of root, stem, leaf vein, and other leaf blade tissues.

Minarik & Shive have added to the contradictory data concerning the boron-calcium relationship. They grew soybeans in solutions with boron concentrations ranging from 0 to 10 p.p.m. Up to a concentration of 0.05 p.p.m., which gives maximum total fresh weight, there was a direct relationship between boron concentration, calcium content, and yield. Satisfactory yields with increasing boron concentrations were obtained up to and including 1.0 p.p.m. The maximum yield (at 0.05 p.p.m.) was 118.1 gm.; that at 0.5 p.p.m., 94.8 gm.; and that at 1.0 p.p.m., 102.2 gm. The calcium content per gram of fresh leaf tissue decreased from 4.5 mg. for a boron concentration of 0.05 p.p.m. to 3.5 mg. for 1.0 p.p.m. concentration. Leaves grown at the next two increasing boron concentrations (2.5 and 5.0 p.p.m.), where there were definite decreases in yield, had calcium contents of 4.2 and 3.6 mg. calcium per gm. fresh weight. From this result the conclusion of these investigators, that toxicity as well as deficiency results in decreased calcium content, does not seem justified. The moisture content was observed to be inversely proportional to the boron concentration from 0 to 5 p.p.m. in the substrate. Contrary to the general trend, however, the moisture content of fresh leaf tissue grown with 5.0 and 10.0 p.p.m. boron concentration increased from 73 to 80 per cent. These results were taken to support the hypothesis that "boron may be a regulator of water absorption by plasma colloids." As is often the case in studies of this type, it is impossible to determine whether the effects are directly or indirectly attributable to boron.

Calcium.—Gauch reported interesting results on the response of the bean plant to calcium deficiency. He used a nutrient solution of the same composition as that used by Nightingale *et al.* for culturing tomatoes. In accord with their results for tomatoes, he found that bean plants also failed to absorb nitrate from the solution in the ab-

sence of calcium. When the concentrations of the solutions containing calcium were changed, and when sodium nitrate was substituted for potassium nitrate in the calcium-free solutions, the plants made much better growth and absorbed considerable amounts of nitrate ion even in the absence of calcium.

Gauch has added also to our knowledge of the effect on the nutrition of plants of various ratios of magnesium to calcium. His results emphasize the importance of total concentration in such studies. With a constant molar concentration of 0.009 for potassium dihydrogen phosphate and of 0.026 for potassium nitrate he arranged three series of calcium nitrate concentrations, 0.00018, 0.0018, and 0.018 mols per liter. With these he varied the magnesium sulfate additions so that the Mg/Ca ratios were as follows: 1, 10, 20, 30, 40, and 50. Judging from the photographs of plants taken seven days after planting, there was very little injury at the lowest calcium nitrate concentration with the highest Mg/Ca ratio, 50, and apparently none with a ratio of 40. With the second calcium nitrate concentration there appeared to be a fairly definite decrease in growth, with a Mg/Ca ratio of 20. At the highest calcium nitrate concentration, only the Mg/Ca ratio of 1 appeared to be noninjurious. Gauch pointed out that the plants in most of the third series were killed because of the high salt concentration. Thus at the Mg/Ca ratio of 10, the total molar concentration was 0.2, while that of the low calcium nitrate series was 0.044 for the Mg/Ca ratio of 50. In the former case the plants appeared to have died before making appreciable growth, while in the latter the plants appeared only slightly smaller than for the lower ratios of the series. He found no Mg/Ca or K/Ca ratios that were optimum over a range of concentrations of salts.

Beneficial effects of very small amounts of calcium on roots of *Pisum sativum* in solution cultures were reported by Sorokin & Sommer. Since their first paper in 1929 there has been, to the reviewer's knowledge, no other work in which an effort has been made to exclude traces of calcium that might be added to the culture solution as a contaminant. As in their earlier work, specially purified salts were used. The water employed in this experiment was redistilled from pyrex. The original calcium concentrations of the solutions were 0, 0.06, 0.125, 0.25, and 314 (control) p.p.m. The solutions, two liters per culture of two plants, were not renewed during the experiment. There was very little difference in the development of the shoots of the plants grown without calcium and at the low calcium concen-

trations. Abnormal mitotic pictures (polyploid and constricted nuclei and binucleate cells) were observed in preparations of root tips as follows: no calcium, abnormal in two to five days; 0.06 p.p.m. calcium, normal at seven and abnormal at fifteen days; 0.125 p.p.m. calcium, normal at fifteen days and abnormal at twenty days; 0.25 p.p.m. calcium, normal at the end of the experiment, four weeks. Failure of development of plants of the 0.25 p.p.m. series may therefore be attributed to functions of calcium not demonstrated in the cytological picture. These investigators suggest dividing the complex reactions of the cells and tissues to the absence of calcium into two groups, those which may be induced by other agencies also, and those peculiar to calcium deficiency. Polyploidy, the formation of binucleate cells, and the premature development of tracheal elements and of lateral primordia are considered attributable to agencies affecting viscosity, surface tension, and, therefore, to include the effect of the calcium in physical-chemical systems. The suppression of mitosis in the absence of calcium and the gradual appearance of aberrant types of division as calcium is removed from the solution are suggested as indicative of a special function of calcium in the protoplast. As in the work reported in their earlier paper, they found no evidence to support the theory that the absence of calcium affects adversely the formation of the middle lamella. The cell walls under these conditions were found never to be underdeveloped, nor to be disintegrating unless infection was evident. Tissues formed under calcium-deficient conditions, were, however, susceptible to invasion by microorganisms.

Chandler studied the calcium content of the foliage of five species of forest trees. He found that in all cases, when expressed either on a percentage or absolute amount basis, the calcium content increased progressively throughout the growing season. The calcium content of evergreen trees increased throughout the growing season but remained fairly constant during the winter months. The longer the foliage of a single species remained on the tree, the greater was its calcium content. On the basis of foliar calcium analyses of twenty-seven forest trees, he classified these trees in three groups according to whether the calcium content of the mature leaves was greater than 2 per cent, between 1 and 2 per cent, or less than 1 per cent.

Iron.—Chapman, Liebig & Vanselow presented evidence that an iron chlorosis which they observed in citrus was due to the presence of an excess of zinc in the nutrient media. Analyses for iron in the various parts of both healthy and chlorotic lemon plants indicated that

the translocation of the iron from the exterior root cells into the vascular system was impeded under these conditions of chlorosis. Thus they found more iron in the leaves, stems, interior roots, and fine roots of green plants but more in the root bark of chlorotic plants. Considering these results and the conclusions of other investigators, they suggest that, assuming iron to be translocated in the ferrous form, zinc may in some way affect the state of oxidation of the iron in a manner similar to that assumed for manganese or copper when iron chlorosis is attributed to excesses of these elements.

Magnesium.—In a study of photosynthesis by *Chlorella,* Kennedy confirmed the observations of Fleischer that an increased chlorophyll concentration caused by the addition of magnesium did not give a corresponding increase in the rate of photosynthesis such as is observed when the chlorophyll content is increased by other means. By use of flashing light he procured evidence that magnesium deficiency is associated with a lowering of the Blackman reaction. By increasing the dark period, he was able to greatly increase photosynthesis per flash per gram of chlorophyll in the case of magnesium deficiency, but obtained no corresponding increase in the case of iron-deficient cells, or for cells grown in full nutrient media.

Manganese.—Burström working with wheat roots, and Noack & Pirson with *Chlorella,* both found manganese to be important in the assimilation of nitrate. Results of Burström's work indicate that this element does not affect nitrate absorption, but is important in the assimilation of both nitrate and nitrite. He found also an inhibiting effect of salts of lithium, sodium, magnesium, calcium, strontium, barium, and heavy metals as sulfates, carbonates, and phosphates. As a working hypothesis, he suggests that, to be effective, the manganese must be adsorbed on the protoplasm, and that the inhibitive action of other cations may be one of displacement of the manganese. Noack & Pirson studied the effect of both iron and manganese on nitrogen assimilation. Solutions with ammonium or nitrate ion with and without glucose and with and without manganese or iron were compared. Iron was without effect, but under some conditions manganese stimulated growth very considerably. The results are here tabulated:

1. $\begin{cases} NO_3 - \text{glucose} + Mn \\ NH_4 - \text{glucose} + Mn \end{cases}$ Growth rate low and the same. Increase noticeable after 3 days.

2. $\begin{cases} NO_3 - \text{glucose} - Mn \\ NH_4 - \text{glucose} - Mn \end{cases}$ Growth rate low and the same (lower than above). Increase noticeable after 5 days.

3. $\begin{cases} NO_3 + glucose + Mn \\ NH_4 + glucose + Mn \end{cases}$ Early growth rapid and the same until the NH$_4$ cultures developed an injurious H-ion concentration. Increase noticeable at 4 days.

4. $NH_4 + glucose - Mn$ Early growth rapid until injurious H-ion concentration developed. Increase in growth noted at 5 days.

5. $NO_3 + glucose - Mn$ Increase in growth was not evident until the eighth day. After this the rate was apparently the same as that for nitrate + glucose + manganese.

The reason why organisms would grow normally after being so severely injured by manganese deficiency as those with nitrate and glucose but without manganese appeared to be, was not determined. It was suggested, however, that it might be because of a substitution of the manganese effect by that of some other metal, perhaps iron, as was noted by Maschmann. Maschmann reported success in substituting an iron-containing for a manganese-containing enzyme in connection with work on dipeptidase of certain anaerobic bacteria.

Working with wheat roots, both with and without removal of the tops, Lundegårdh obtained an increase in oxygen consumption in the presence of manganese. Manganese as chloride or sulfate was used in concentrations of 0.0002 to 0.0005 M. Most of the work was done in solutions of 0.001 M potassium chloride. Similar results were obtained with 0.001 and 0.002 M potassium nitrate. He found this increase in oxygen consumption true only for what he terms *Grundatmung* in contrast with what he considers necessary for the uptake of ions. He found no influence of the presence of manganese on the uptake of ions, chloride, phosphate, or nitrate. He reported a liberation of manganese and calcium by potassium ions when roots were placed in solutions of potassium bromide and chloride. The amount of calcium and manganese replaced was increased by the addition of 0.005 M lead chloride or ascorbic acid. Marked differences in potential were obtained with manganese-free as compared with normal roots.

Nitrogen.—Chapman & Liebig found that concentrations of nitrate nitrogen of 6 to 7 p.p.m., when maintained, were adequate for rapid development of citrus plants even in the presence of relatively high concentrations of chloride or sulfate ions. The varying amounts of nitrogen were added as mixtures of potassium, calcium, and magnesium nitrate of such proportions that the equivalent amounts of these

cations were kept constant. Concentrations of nitrogen over and above that required for maximum vegetative growth were without effect upon the vegetative growth of the tops, but the ratio of top to root increased with increasing nitrate concentration. Distinct impairment of growth of plants in solutions with a nitrate concentration of 840 p.p.m. was (as indicated by leaf analyses) due probably to high potassium absorption rather than to nitrogen injury. The absence of the expected decrease in nitrate absorption due to large amounts of chloride or sulfate was explained on the grounds that the increased absorption of potassium over calcium under these conditions had a favorable effect on the absorption of the nitrate ion and thus offset any potentially depressing effect of the sulfate or chloride. When chlorides or sulfates were used, they were added as mixtures of the potassium, calcium, and magnesium salts in such proportions that the equivalent amounts of these cations were kept constant. It was decided, therefore, that, as pointed out by Vanselow in some of his unpublished work, the increase in absorption of potassium over calcium which occurred at higher concentrations was a mass action effect. This complicating mass action effect, occurring when equivalent amounts of cations of different valences are used in such comparative work has, to the reviewer's knowledge, not been noted before. It emphasizes both the difficulty in choosing between molar and equivalent concentrations for certain types of work and the importance of such considerations in comparing the results of different investigators. Chapman & Liebig found no depressing effect on phosphate absorption due to increased nitrate concentration. An increased amount of phosphorus accumulated in the tissue at certain stages of nitrogen starvation appeared to be due to retarded growth.

Phosphorus.—A probable relationship between phosphorus and manganese deficiencies in citrus grown in calcareous soils of low phosphate availability is described by Chapman, Liebig & Vanselow. New leaves, formed after phosphorus deficiency symptoms (including leaf abscission) had developed, showed manganese deficiency symptoms, while trees receiving phosphate grew normally. Painting the affected leaves with manganese chloride solution brought about recovery. It is interesting to note that one of the reasons suggested for the occurrence of manganese deficiency in such cases is a decreased absorption due to poor root development caused by phosphorus deficiency. A search of the literature reveals much evidence that the root/top ratio increases with decreasing phosphate concentration. This has been em-

phasized by Sommer as a result of solution culture work; and also by Goedewaagen, who obtained similar results with soils. The tables given both by Goedewaagen and by Sommer show cases in which the weights of the roots were greater at the lower phosphate concentrations while the tops showed a reverse relationship. The phosphate concentrations Sommer used were from 12.8 to 0.1 p.p.m., much lower than most investigators in solution culture work employ.

Potassium.—Two papers by Richards & Shih suggest reasons for the contradictory data reported by different investigators on the relationship between potassium deficiency and succulence. For the experiment from which they secured the data discussed in these papers, barley was grown in solutions of ten basal nutrient types at three potassium levels, in all, twenty-two salt combinations. These basal nutrients consisted of five different combinations of sodium and calcium, together with two levels of phosphorus. Samples were taken at three different times during vegetative growth, and determinations of water content of the leaf and stem fractions, and the percentage composition of potassium, calcium, and phosphorus in the green leaf were made. The first paper reports the relationship of water content of the barley leaves to potassium level in the nutrient media and the effects of the concentrations of other ions present. Their results, statistically treated, indicate the following relationships:

Increasing deficiency of potassium leads in general to progressively greater succulence. . . . . Water content increases markedly with potassium deficiency at the high sodium levels and less markedly in nutrients containing medium levels of sodium together with calcium, while at high calcium levels there is a minimum of succulence at about the K_3 [intermediate potassium] level. . . . . Reduction . . . . of phosphorus level [to one fifth] leads in general to a lowering of water content . . . . The decrease in succulence associated with reduced phosphorus supply is negligible at high potassium levels, but increases markedly as the potassium supply is lowered. . . . . The differences in succulence associated with varying phosphorus supply are least at high calcium levels . . . . and greatest in series . . . . containing both sodium and calcium [at medium levels].

The second paper discusses the relationship between content of potassium, sodium, calcium and phosphorus in the leaf, and water content. They failed to find any appreciable effect of potassium content as such on succulence.

Potassium is the only element of those whose effects were measured which shows no appreciable relationship to succulence between treatments, though in the interaction of treatments with times of sampling small and complex but highly significant relationships are found.

According to their results, sodium is most and phosphorus next important in effect on leaf water content. Sodium and phosphorus effects were highly correlated, and it appeared that, in general, phosphorus and calcium effects accompanied sodium effects. The authors discuss at length the probable effect on succulence of variations in carbohydrate level among various treatments (carbohydrate determinations were not made in this study) and also the age of the plant.

They are held to account for the apparent facts that water content becomes more sensitive to internal concentrations of the elements with (1) decreasing potassium supply, and (2) increasing age. It is concluded that the greatest effect of carbohydrate on succulence is that exerted on cell-wall extensibility as determined by thickness of wall; for a given suction pressure a thin-walled cell will absorb more water than a thick-walled, more rigid cell.

It is suggested that

The decrease in water content with reduced potassium supply, often reported in the literature, appears to be, in the main, a consequence of increased carbohydrate level, direct effects of potassium content being subordinate in magnitude.

More data concerning the effect of potassium concentration on carbohydrate and nitrogen metabolism of the tomato plant are presented by Wall (2, 3). The concentrations of potassium used were 0, 2.5, 11.0, 22.0, 45.0, and 175 p.p.m. There was increase in growth with increments of potassium to 45 p.p.m. Two types of potassium deficiency symptoms were noted: (a) early in the course of the experiment there was a marked stunting of the plants which became hard and yellow; and (b) a later effect, when the plants again began to grow, they became at this time green and soft. The first stage was accompanied by a high carbohydrate content in the plants cultured in solutions of low potassium content. In the second stage the carbohydrate was greatly diminished. These observations are interesting in connection with the conclusions of Richards & Shih mentioned above. Potassium-deficient plants had a higher soluble organic and total nitrogen content than did plants with a sufficient amount of this element. The potassium content of plants increased slowly with increasing potassium in the nutrient solution up to 22 p.p.m. Sharp increases were noted for plants grown in solutions with 45 and 175 p.p.m. of potassium. In comparing nitrate and ammonium nitrogen when potassium was deficient, not only was better growth found with plants supplied with nitrate, but much more severe and rapid injury in potassium-deficient cultures when the ammonium ion was employed. A rapid breakdown of leaf tissue appeared to be due to high internal

ammonium ion concentrations in the potassium-deficient plants. In both cases ammonium, amide, and amino acid nitrogen increased and protein nitrogen decreased when potassium was deficient.

Day reported that the maximal amount of starch in the leaves of tobacco plants was less when the plants suffered from potassium deficiency than when well supplied with this element, and that decreased water content was associated with potassium deficiency. The plants were grown in solutions which differed only in that the potassium-deficient solutions contained 0.497 gm. of sodium dihydrogen phosphate monohydrate instead of 0.442 gm. of potassium dihydrogen phosphate per liter. The determinations were made on plants toward the end of their vegetative period and the samples were taken at 3 P.M. on sunny days.

Working with citrus trees, Chapman, Liebig & Vanselow observed toxicity in potassium-deficient cultures with a boron concentration (1 p.p.m.) that was not injurious in complete nutrient solution nor in potassium-deficient solutions to which 20 milliequivalents of sodium had been added. Growth repression was the same in the two potassium-deficient solutions, but the potassium deficiency symptoms did not develop as early nor as acutely for the plants grown in solutions containing a high sodium content.

Silicon.—Wagner has made an important contribution concerning the importance of silicon to plant growth. Working with redistilled water and specially analyzed salts he obtained striking results with a number of plants. Young plants grew equally well with and without silicon for from four to six weeks. After the onset of deficiency symptoms, the abnormalities of the plants grown without silicon increased with time. For oats and rice there was a decrease in the number of shoots and less root development when silicon was deficient. Plants grown in solutions to which silicon had been added were rougher, harder, and firmer and stood upright, while those grown without silicon bent over and their tips hung down. In rice plants the most striking symptoms appeared after tillering. Bright yellow stripes appeared between the veins of the middle part of the leaves. These, after drying, had a browner color and extended more or less over the whole width of the leaves. The leaves were often rolled and spirally twisted. Cucumbers, besides growth depression in silicon-deficient solutions, showed abnormal conditions of the leaves. There were irregularly defined necrotic spots distributed over the whole leaf surface. The edges of the leaves rolled toward the under side and had a stiff appearance.

The leaves were brittle and in general were of lighter color than where silicon was present. The symptoms for oats appeared later than for rice and cucumber. Plants without silicon bore little grain, and that small, and ripened early. Corn, tomatoes, bushbeans, tobacco, and barley grew less in the absence of silicon but showed no special deficiency symptoms. The depression in growth was greater with rice than with cucumbers, tobacco, and oats. Barley plants without silicon succumbed to mildew so early that comparison of nutritional injury could not be valid. The transpiration rate of plants without silicon was much greater than that of plants with silicon. Experiments concerning the partial substitution of phosphorus by silicon gave negative results. The criterion, however, was the result of a comparison of plants grown at two different phosphorus levels (0.7 gm. per liter and 0.35 gm. per liter) with and without silicon in the solution. Different results might have been obtained if, instead of 0.35 gm. phosphorus, less or none had been used. The presence of silicon in culture solutions increased the resistance of the plants to mildew. An increase in incubation time and a decrease in the number of spots on the leaves resulted when silicic acid was added to pot cultures of cucumbers in soil of a marshy type. There appeared to be no relationship between mildew resistance and silicon content of resistant and nonresistant varieties of barley.

Zinc.—Eltinge & Reed studied the effects of zinc deficiency on tomato roots. For this study they used roots cultured by three methods: (*a*) from plants grown by the ordinary solution culture method, with the use of purified salts and pyrex containers; (*b*) from sterile seedlings grown on agar slants; and (*c*) sterile root tips in hanging drops. These roots in the absence of zinc, were found to have swellings on which appeared tufts of root hairs, many of which were crooked and showed various abnormalities. Secondary roots tended to develop at the nodular enlargements, and the tip of the main root often died. Elongation was continued by the growth of a cluster of secondary roots which broke through near the dead tip. Microscopic examination showed profound derangement of the meristematic region, and abnormal metabolic products in the cell vacuole were reported.

Scandium.—Steinberg (1, 2) has added to his interesting and important work concerning those elements necessary in only small amounts for plant growth. The addition of scandium (2) to cultures of *Aspergillus niger* in solutions containing glycerol as the source of

carbon doubled the yield. He points out that this is an example of biological specificity since its nonaddition was without effect with other sources of carbon. He (1, 2) also obtained further proof of the necessity of iron, zinc, copper, molybdenum, and gallium for the growth of this organism.

In working on the problem of efficiency of photosynthesis with *Chlorella,* Emerson & Lewis found that the addition of an extra amount of ferric sulfate greatly increased the efficiency of cells grown in a given medium and that the improvement was not due to the iron itself, but to impurities contained in considerable amounts in the particular iron salt. In the course of their study they employed two groups of elements (Arnon classification), one, A5, containing those "micro-elements" known to be essential for the normal development of certain higher plants (boron, manganese, zinc, copper, and molybdenum), and the other, B6, including those elements not known to be essential but known to be definitely beneficial when added as a group (titanium, vanadium, tungsten, chromium, cobalt, and nickel). The change of designating numbers from A4 and B7 (Arnon) to A5 and B6 was made because of the transference of molybdenum to the A group when molybdenum was found necessary for the normal growth of certain plants (Arnon & Stout). Emerson & Lewis found that the addition of A5 improved the photosynthetic efficiency of *Chlorella* 85 per cent and that of B6, 29 per cent. Used in combination, the quantum yield was increased more than 100 per cent.

LITERATURE CITED

ARNON, D. I., *Am. J. Botany,* **25**, 322 (1938)

ARNON, D. I., AND STOUT, P. R., *Plant Physiol.,* **14**, 599 (1939)

BROYER, T. C., AND OVERSTREET, R., *Am. J. Botany,* **27**, 425 (1940)

BURSTRÖM, H., *Planta,* **30**, 129 (1939)

CHANDLER, JR., R. F., *Cornell Univ. Agr. Expt. Sta. Mem.,* 228 (1939)

CHAPMAN, H. D., AND LIEBIG, JR., G. F., *Hilgardia,* **13**, 141 (1940)

CHAPMAN, H. D., LIEBIG, JR., G. F., AND VANSELOW, A. P., *Proc. Soil Sci. Soc. Am.,* **4**, 196 (1940)

DARROW, R. A., *Botan. Gaz.,* **101**, 109 (1939)

DAY, D., *Plant Physiol.,* **15**, 367 (1940)

EATON, F., *Botan. Gaz.,* **101**, 700 (1940)

ELTINGE, E. T., AND REED, H. S., *Am. J. Botany,* **27**, 331 (1940)

EMERSON, R., AND LEWIS, C. M., *Am. J. Botany,* **26**, 808 (1939)

FLEISCHER, W. E., *J. Gen. Physiol.,* **18**, 573 (1935)

GAUCH, H., *Plant Physiol.,* **15**, 1 (1940)

GOEDEWAAGEN, M. A. J., *Soil Sci.,* **44**, 185 (1937)

HOAGLAND, D. R., AND BROYER, T. C., *Am. J. Botany,* **27**, 173 (1940)

KENNEDY, JR., S. R., *Am. J. Botany,* **27**, 68 (1940)

LUNDEGÅRDH, H., *Planta,* **29**, 419 (1939)

MASCHMANN, E., *Naturwissenschaften,* **27**, 276 (1939)

MINARIK, C. E., AND SHIVE, J. W., *Am. J. Botany,* **26**, 827 (1939)

NIGHTINGALE, G. T., ADDOMS, R. M., ROBBINS, W. R., AND SCHERMERHORN, L. G., *Plant Physiol.,* **6**, 605 (1931)

NOACK, K., AND PIRSON, A., *Ber. deut. botan. Ges.,* **57**, 442 (1939)

OVERSTREET, R., AND BROYER, T. C., *Proc. Natl. Acad. Sci. U.S.,* **26**, 16 (1940)

RICHARDS, F. J., AND SHIH, S., (1), *Ann. Botany,* **4**, 165 (1940)

RICHARDS, F. J., AND SHIH, S., (2), *Ann. Botany,* **4**, 403 (1940)

SCHLENKER, F. S., *Am. J. Botany,* **27**, 525 (1940)

SOMMER, A. L., *J. Agr. Research,* **52**, 133 (1936)

SOROKIN, H., AND SOMMER, A. L., *Am. J. Botany,* **27**, 308 (1940)

STEINBERG, R. A., (1), *J. Agr. Research,* **59**, 731 (1939)

STEINBERG, R. A., (2), *J. Agr. Research,* **59**, 749 (1939)

STILES, W., AND SKELDING, A. D., *Ann. Botany,* **4**, 329 (1940)

STEWARD, F. C., AND PRESTON, C., *Plant Physiol.,* **15**, 23 (1940)

STEWARD, F. C., STOUT, P. R., AND PRESTON, C., *Plant Physiol.,* **15**, 409 (1940)

VANSELOW, A. P. (Unpublished work)

WAGNER, F., *Phytopath. Z.,* **12**, 427 (1940)

WALL, M. E., (1), *Soil Sci.,* **47**, 143 (1939)

WALL, M. E., (2), *Soil Sci.,* **49**, 315 (1940)

WALL, M. E., (3), *Soil Sci.,* **49**, 393 (1940)

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PLANT GROWTH SUBSTANCES

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The review which follows is not intended to be inclusive. The
numerous recent contributions on the auxins are omitted entirely, and
part only of the literature on other groups of growth substances is
considered. Particular attention is devoted to the vitamins and vita-
min-like growth substances. The reader's attention is directed to the
extensive reviews by Koser & Saunders (1), Schopfer (2), Janke (3),
to shorter articles appearing in *Chronica Botanica* and to the *Proceed-
ings of the Third International Congress for Microbiology.*

There is no unanimity in terminology. The growth substances
are referred to in the current literature as plant hormones, vitamins,
ergones, auxithals, nutrilites, growth factors, accessory growth fac-
tors, growth activators, and so on. Bottomley's original term, auxi-
mone, rarely appears. A growth substance, in the specialized sense
in which the term is now used, refers to a definite organic substance
minute amounts of which have a materially favorable effect upon
plant development. Many growth substances (perhaps all) are specific
and indispensable.

Methods of Investigation

In experiments with growth substances details of technique are
quite important because of the minute amounts of these substances
which are effective and because of their wide distribution in products
of natural origin. It is desirable to emphasize the following points
although they are well known to most workers in the field.

Cleanliness of glassware.—It hardly seems necessary to refer to
the need of strict cleanliness of glassware and other utensils used.
However, White (4) has recently demonstrated the errors which ig-
norance of methods of cleaning glassware or carelessness may cause.

The basic medium and general conditions of growth.—In tests for
growth substances the basic medium should be adequate in all other
respects and approach as nearly as possible that most suitable for the
organism in question. Trace elements or minor mineral elements de-
serve special attention (5), and the possibility that supplements cor-

rect toxicity rather than a deficiency must be considered. Environmental conditions affect growth substance deficiencies though relatively little study has been devoted to this aspect of the problem. Robbins & Kavanagh (6) demonstrated that the ability of *Pythium Butleri* to synthesize thiamin is affected by the salt content of the medium. Robbins (7) found temperature to play an important role in the synthesis of unidentified growth substances by *Phycomyces*. Oxygen supply influences the need for uracil by *Staphylococcus aureus* (8). *Rhodotorula sanniei* grows as well on a glycerine solution as on a glucose solution supplemented with pyrimidine[1] (9). The rate of growth probably affects the demand for a particular growth substance, and deficiencies may appear under rapid growth conditions which are not evident when the organism grows slowly.

Purity of chemicals.—The purity of chemicals, especially those of natural origin, must be considered. Carbohydrates, even those of chemically pure grade, may contain growth substances (10, 11, 12). Maltose is especially likely to be contaminated (13). Agar (14), cotton (15), gelatine (16), and probably some grades of filter paper contain significant amounts of growth substances. Crude asparagine must be purified.

Methods of sterilization.—The mode of sterilizing the medium to be tested may affect the results. Sterilization by heat may inactivate a growth substance (17), and filtration may result in the adsorption of some or all of it.

Storage of growth factors in cells.—A reserve of growth factor stored in the organism being investigated may compensate for a deficiency of a growth substance in the medium. Successive passages in a medium free of growth substances will overcome this difficulty (18).

Multiple deficiencies.—The lack of growth factors, besides the one being examined, may cause misinterpretations of results on certain organisms. Little development may occur when single growth substances are used as supplements to the basic medium while a combination of two or more may produce a marked effect. Williams & Saunders (19) have demonstrated this for yeast; Hawker (20), for *Melanospora;* and Robbins & Schmidt (21) and others for excised tomato roots.

Heterotrophy and autotrophy.—The degree to which an organism

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[1] The terms thiazole and pyrimidine are used in this paper to refer to the two intermediates of thiamin.

is heterotrophic or autotrophic for one or more growth substances is also of importance in the interpretation of results.

Some authors refer to a growth substance as "essential" when the organism will not grow unless some of the particular growth substance is present in the medium; as "stimulatory" when the organism grows in a medium lacking the growth substance in question but develops better when some is present; and as "non-essential" or "not required" when the organism grows as well in the presence as in the absence of the growth substance. It is possible that the implications of this terminology are correct and that an organism may be able to grow without a particular growth substance while being stimulated by its presence, and that a particular growth substance plays an important role in the physiology of one organism and not in that of another. However, accumulating evidence indicates that all three classes are probably necessary for normal metabolism, but that the organism itself synthesizes none of the "essential" growth substance and is completely heterotrophic in that regard, synthesizes some but inadequate amounts of the "stimulatory" ones, and is entirely autotrophic for those which are "non-essential" or "not required" (22).

Differences between nearly related organisms.—Degree of heterotrophy for particular growth substances may differ with the species, race, or strain. This caused some confusion in the earlier work with yeasts (23). Hawker found some strains of *Melanospora destruens* to be completely heterotrophic for biotin while others grew and fruited on media lacking biotin and thiamin. Numerous similar examples have been reported for the bacteria.

Source of the growth substance.—Synthetic preparations are to be preferred. Crystalline products from natural sources may be pure or may contain traces of other growth substances. The results from concentrates are frequently difficult to interpret and always need confirmation from experiments with the pure compounds when available.

Amount of growth substance.—The quantity of growth substances supplied to an organism or to a culture appears in general to be more important than its concentration. Many investigators express the quantity furnished per milliliter of medium. Comparisons of effects on the basis of concentrations when the volumes differ materially may lead to erroneous conclusions.

Bioassays.—Plants are playing an increasingly important role as test organisms for the isolation of specific growth substances and for their quantitative determination. The use of a strain of yeast in the

isolation of biotin by Kögl & Tönnis (24) is a fine example of the application of a plant bioassay. Such assays, employed for the quantitative estimation of vitamins, are subject to error because the organism employed *(Phycomyces)* may respond to the decomposition products of the vitamin as well as to the vitamin as such; or it may respond to nearly related compounds less available for the animal than for the plant used in the assay (25); or it may fail to respond to compounds available for animals (18). For biotin, yeast and *Ashbya gossypii* (26, 27) have both been used.

For pantothenic acid as such, assay methods using *Lactobacillus casei* or *Streptococcus lactis*-125 have proved useful (28). For thiamin and its intermediates, *Phycomyces Blakesleeanus* (29, 30, 31), and yeasts have been used. For thiamin as such, *Phytophthora cinnamomi* was used by Bonner & Buchman (32). For riboflavin, *Lactobacillus casei* or *Bacillus lactis acidi* have been employed (18). The growth of yeast has been suggested as a means of estimating pyridoxin (33).

FUNCTION OF GROWTH SUBSTANCES

Two general concepts of growth substances, not mutually exclusive, seem to underlie the interpretations of observations by various investigators. One concept emphasizes the relation of a given substance to the production of observable morphological changes in the organism and results in the description of root hormones, flower hormones, and hormones for cell division, cell elongation, etc. The other concept assumes that the particular growth substance plays a specific role in the metabolism of the organism and that any morphological change is incidental to this function. Growth substances probably do not all function in the same way. Many are parts of enzyme systems or precursors of such parts (34); some are building stones of nonenzymatic constituents of the organism, for example nucleoproteins; and it is possible that others may function in other ways, for example in maintaining surface layers or influencing permeability.

GROWTH SUBSTANCES

Thiamin (vitamin B_1, aneurin).—The importance of thiamin as a growth substance for bacteria, yeasts, filamentous fungi, and the excised roots of higher plants has been further confirmed; and the number of organisms which show thiamin deficiencies has been con-

siderably increased (20, 35 to 43). Further evidence has been presented that all degrees of heterotrophy exist, ranging from organisms which require supplements of thiamin as such to those which are partially or completely autotrophic and including organisms incapable of making either intermediate, or able to synthesize the pyrimidine half of the molecule or the thiazole portion (22, 41). Additional contributions to the specificity of thiamin have appeared (25, 25a, 31, 44, 44a, 45). Of particular interest in this regard is the concept of isosterism which requires further exploration (46, 47).

Bonner & Buchman (32) demonstrated that actively growing *Phycomyces* synthesized thiamin from a mixture of the thiazole and pyrimidine intermediates and that the resting mycelium destroyed it. The breakdown of the vitamin *in vivo* resulted in an inactivation or destruction of the thiazole portion of the molecule leaving the pyrimidine half intact and available for recombination with additional thiazole if present. The destruction of the thiazole appeared to occur while it was in the form of thiamin and not as free thiazole. Addition of excess pyrimidine to a solution in which the molar ratio of the intermediates was unity resulted in no increased growth while addition of excess thiazole materially increased growth. When the amount of pyrimidine was small the addition of a ten-fold excess of thiazole doubled the growth as compared with that obtained when the thiazole was present in quantities molecularly equivalent to the pyrimidine. These findings, which are contrary to those of Robbins & Kavanagh, are important from the standpoint of our knowledge of the metabolism of the vitamin, and because of their relation to bioassays with *Phycomyces* for thiamin and its intermediates.

Buchman & Richardson (48) synthesized an amino acid, β-(4-methylthiazolyl-5)-alanine, which they considered might be the precursor of thiazole *in vivo;* and Bonner & Buchman (49) found excised pea roots able to convert the amino acid to thiazole. Robbins (50) reported the amino acid to be as effective as thiazole for excised tomato roots and to be effective for *Phycomyces* also if enough was used.

Robbins (51) found no positive correlation with hybrid vigor of the thiamin content of grains of inbred maize and the heterotic offspring. Bonner (40) found excised roots of flax and clover able to synthesize small amounts of thiamin, and McClary (52) reported that excised maize roots grown on an agar medium synthesized thiamin. Blumer & Schopfer (45) in an extensive study found *Ustilago scabiosae* to show a thiamin deficiency. Mixtures of the pyrimidine and

thiazole intermediates replaced thiamin when used in relatively large quantities, but optimal development was obtained also if either intermediate was present in small amount and the other in excess. They considered *Ustilago* to resemble *Phycomyces* in its relation to thiamin but to be less efficient in forming the thiamin molecule from the intermediates.

Lilly & Leonian (53) found thiamin to be present in the soil; but whether it is present there as free thiamin or in the bodies of organisms is not clear; and Bonner & Greene (54) found measurable amounts in manure. By means of the *Phycomyces* assay Rytz (55) found thiamin (or its intermediates) to be highest per unit dry weight in the leaves and least in the roots of pea. Per plant the material active for *Phycomyces* was greatest during the first fifteen days of growth.

Burkholder & McVeigh (56) reported on the effect of temperature, source and amount of nitrogen, hydrion concentration, and other factors on the growth of *Phycomyces* with special reference to the use of that organism for the bioassay of thiamin, and in a later paper (57) reported analyses of the thiamin content of lines of maize, grown in various mineral solutions, and of other plants. Light seems to be associated with the synthesis of thiamin by tomato plants and in girdled plants it accumulates above the girdled portion of the stem indicating that it is produced in the leaves and conducted through the phloem (58). It is not clear whether the effect of light is direct or indirect or associated with the chlorophyll in the leaves; certainly many plants (bacteria and fungi) which lack chlorophyll synthesize thiamin in the dark.

Bonner (59) and Bonner & Greene (54) reported large increases in the growth of some kinds of green plants when thiamin was added to the nutrient medium in which the plants were grown. Bonner & Bonner (60) also found thiamin as well as various other growth substances (nicotinic acid, pyridoxin, adenine, uric acid, and estrone) beneficial to entire plants. Similar reports have appeared in various horticultural journals, and much interest developed in the use of thiamin in horticultural practice. However, Arnon (61) and Hamner (62) found no benefit from thiamin for plants grown from seeds in nutrient solutions. It appears that the application of thiamin to intact higher plants is without benefit or the conditions under which it is beneficial are ill-defined. A somewhat similar situation appears to exist for the effect of thiamin on the rooting of cuttings (63).

Pantothenic acid.—First discovered by R. J. Williams, pantothenic

acid has now been synthesized (64) and is available in the form of its calcium salts. It is α,γ-dihydroxy-β,β-dimethylbutyryl-β'-alanide and was first obtained by synthesis from the lactone of (+)-α,γ-dihydroxy-β,β-dimethylbutyric acid and β-alanine ethyl ester. Its vitamin activity in chick dermatitis has been recognized by Jukes (65) and by Woolley, Waisman & Elvehjem (66), and for the growth of young rats by Hoffer & Reichstein (67).

It is important for the growth of many strains of yeast (33, 43) and for several bacteria including *Streptococcus lactis, Lactobacillus casei, Bacillus brassicae, Propionibacterium pentosaceum* (68), certain strains of hemolytic streptococci (69, 70) diphtheria bacteria (71), and some types of *Pneumococcus* (72). *Streptococcus lactis* requires pantothenic acid as such (28), but some strains of *Saccharomyces cerevisiae* grow well with β-alanine alone (33, 43, 43a). Mueller & co-workers (71, 71a) found that β-alanine and pantothenic acid both stimulate diphtheria bacteria and that the bacteria synthesize pantothenic acid from β-alanine. No positive reports of benefits to filamentous fungi or excised roots have appeared; those tests which have been made have been negative (2, 29, 39).

It seems probable that plants which do not require a supply of pantothenic acid synthesize adequate amounts from elementary materials and that the requirements for this substance will be found to vary with the organism from complete heterotrophy (*S. lactis*) through partial heterotrophy (yeast) to complete autotrophy.

Pantothenic acid appears to have a considerable degree of specificity. As indicated above it is prepared from the (+) lactone. Pantothenic acid prepared from the (−) lactone is inactive for *Streptococcus lactis* and that from the racemic lactone has about 50 per cent of the activity of the pure substance (64). Pantothenic acid prepared from α-hydroxy-γ-*n*-valerolactone, α-hydroxy-β-methyl-γ-butyrolactone, α-hydroxy-α-methyl-γ-butyrolactone had less than 0.5 per cent of the activity of pure pantothenic acid. Preparations made from erythronic lactone or α-hydroxy-γ-butyrolactone had no activity (73). N-(α,*d*-dihydroxyvaleryl)-β-alanine supports growth of certain hemolytic streptococci (69, 70) but neither it nor N-(α,ε-dihydroxycaproyl)-β-alanine has more than a fraction of 1 per cent of the activity of true pantothenic acid. Availability of N-(α-hydroxy-β,β'-dimethylbutyryl)-β-alanine varies with the organism and cultural conditions but is from 5 to 25 per cent as effective as pantothenic acid.

p-*Aminobenzoic acid.*—Rubbo & Gillespie (74) found that *p*-amino-

benzoic acid and its derivatives act as growth factors for *Clostridium acetobutylicum*. Of especial interest is their suggestion that sulphanilamide is effective by inactivating an essential coenzymic grouping of the susceptible organism and that this grouping is probably *p*-aminobenzoic acid.

Pyridoxin (vitamin B_6, adermin).—Pyridoxin is 2-methyl-3-hydroxy-4,5-hydroxymethyl pyridine. The synthetic product in the form of the hydrochloride can be readily purchased. It appears to be a growth substance for plants although differences exist in the findings of various investigators. Möller (75) found *B. acetylcholini* to require vitamin B_6, with optimum action at 0.4 to 0.6 µg. per ml. It could not be replaced by inositol, asparagine, β-alanine, thiamin, nicotinic acid, pimelic acid, creatine, urea, uric acid, adenine, xanthine, guanine, uracil, or *dl*-methionine. Vitamin B_6 was favorable also to *Streptobacterium plantarum, Bacterium utile* (Henneberg) and a strain of bottom yeast isolated from sauerkraut. Woolley & Hutchings (69) reported a wide variety of hemolytic streptococci to require riboflavin, pantothenic acid, a mixture of amino acids, and vitamin B_6. Vilter & Spies (76) found synthetic pyridoxin in amounts of 0.3 to 1.2 µg. per ml. to stimulate growth and acid production of *Staphylococcus albus* in the presence of nicotinic acid and thiamin. Sinclair (29) reported vitamin B_6 in the presence of thiamin to improve the growth of *Phycomyces Blakesleeanus;* however, Robbins (77) and Jung & Schopfer (78) found vitamin B_6 alone and with other growth factors to be without effect on *Phycomyces*. The latter authors by rat-feeding experiments demonstrated that *Phycomyces* mycelium grown on synthetic media contains about 100 µg. of vitamin B_6 per gram of dry mycelium. Schultz, Atkin & Frey (33) and Eakin & Williams (79) reported pyridoxin as a growth substance for yeast. However, there is considerable difference with different strains of yeast. Williams, Eakin & Snell (43) studied the growth curves of three strains of *Saccharomyces cerevisiae* in media containing different amounts of inositol, biotin, pantothenic acid, and pyridoxin and found vitamin B_6 relatively unimportant. Schultz, Atkin & Frey (42) in a survey of forty-four strains of *S. cerevisiae* and *S. carlsbergensis* established three types: type A, growth increased by thiamin and further increased by thiamin and pyridoxin; type B, growth decreased 50 per cent or less by thiamin and brought back to normal by the further addition of pyridoxin; type C, yeasts decreased 50 per cent or more by thiamin and increased above normal by the further addition of

pyridoxin. Robbins & Schmidt (21) reported vitamin B_6 to be highly beneficial in the presence of thiamin for the growth of excised tomato roots. Robbins (80) found the excised roots of one inbred tomato to show little response to vitamin B_6 while those of another inbred and the F_1 heterotic hybrid showed considerable response. Bonner & Devirian (39) reported vitamin B_6 to be without effect on the growth of excised roots of peas, radish, and flax but obtained positive results with tomato. Bonner (40) found no effect on excised roots of alfalfa, clover, or cotton but beneficial results with excised roots of *Datura stramonium*, carrot, and five strains of tomato. Stoutemyer (81) found the rooting of the cuttings of some kinds of plants improved by applications of vitamin B_6 while others showed no response. White (4) concluded that pyridoxin was not beneficial to the growth of excised tomato roots.

Biotin.—Biotin was first isolated in crystalline form and found to be a growth substance for yeast by Kögl & Tönnis (24) and for several filamentous fungi by Kögl & Fries (82). Further work has amply confirmed the importance of biotin for the growth of many bacteria, yeasts, and fungi although investigations have been handicapped by difficulty in obtaining the crystalline material for experimental purposes. Fries (83) in an extensive study of the requirements of various wood-destroying and other fungi found some to show deficiencies for thiamin and others for biotin or biotin and thiamin. Lindeberg (38) found various species of *Marasmius* to fall into two groups, one requiring thiamin for growth, the other thiamin and biotin. Of two strains of *M. perforans* one belonged to the first group and one to the second. Melin & Lindeberg (37) found that thiamin was important for several mycorrhizal fungi and thiamin and biotin for one, *Rhizopogon roseolus*.

Of particular interest is Hawker's (20) discovery that *Melanospora destruens* grows on a medium supplemented with biotin but does not form perithecia while growth and production of perithecia occur if thiamin and biotin are both present. Kögl & van Wagtendonk (84) reported biotin in addition to thiamin and nicotinic acid to be effective with *Staphylococcus pyogenes aureus*. Williams, Eakin & Snell (43) found that of three strains of yeast thiamin is important for one only while pantothenic acid and biotin are important for all three. West & Wilson (85) found biotin concentrates to favor growth of *Rhizobium* but not that of *Phytomonas tumefaciens, Achromabacter radiobacter* or two species of *Azotobacter*. Coenzyme R, first de-

scribed by Allison, Hoover & Burk (86), vitamin H, and biotin have been found to be identical; and a crystalline product with a different melting point and higher activity than that prepared by Kögl has been isolated (87).

Carotenoids.—Moewus (88) reported that living cells of *Chlamydomonas eugametos* washed from Knop's agar medium became motile when placed in an aqueous suspension and exposed to light (either red, yellow, green, blue, or violet). In the dark and with oxygen they became motile when supplied with sugar. In the dark and without oxygen, motility occurred in the presence of filtrates of motile cells or in solutions containing crocetin or its glucoside, crocin. Crocin is active at a dilution of 1 part in 250 trillion which means that one or a very few molecules per cell are effective. Moewus found further that exposure of the cells to blue or violet light developed ability to conjugate, the female gametes requiring shorter exposure than the male gametes. A filtrate from female cells after exposure to blue or violet light induced the formation of male gametes but with continued illumination became ineffective (K_0). A pro sex material (V) was formed in red light and was present in filtrates. On exposure to light V first became effective in inducing the development of female gametes and then male gametes. The pro sex material was identifid as *cis*-crocetin dimethyl ester (V) and the ineffective end product as *trans*-crocetin dimethyl ester (K_0). The production of female and male gametes was found to depend upon the relative amounts of V and K_0. For female gametes three parts of V and one of K_0 were necessary and for male gametes one of V and three of K_0. More of the carotenoids were required to induce conjugation than to induce motility. Confirmation of this striking piece of work and its extension to other organisms are desirable.

Raper (89, 90) has presented evidence for the existence of four substances affecting the formation of sex organs and the directional growth of antheridial branches to the female initials in the heterothallic mold, *Achlya sp.*

Pimelic acid.—Pimelic acid, found by Mueller (91) to be a growth substance for strains of the diphtheria bacillus, has not benefited other plants. Hall (92) found it beneficial to an autotrophic protozoan, *Colpidium campylum.*

Traumatins.—Traumatic acid was isolated and synthesized by English, Bonner & Haagen-Smit (93). It is 1-decene-1,10-dicarboxylic acid. Traumatic acid induces cell extension activity in the parenchym-

atous cells of the mesocarp of the bean pod and the formation of wound periderm in washed discs of potato tuber. It is considered to be the wound hormone postulated by Haberlandt in 1913. Van Overbeek (94) found traumatic acid highly effective in promoting multiplication of species of *Scenedesmus* and other algae.

Riboflavin.—Riboflavin has been shown to be a growth factor for several lactic acid bacteria by Orla-Jensen & co-workers in 1936 (95) and for *Streptococcus hemolyticus* Dochez N Y 5 by Rane & Subbarow in 1938 (96). Woolley & Hutchings (69, 97) have found that three strains of *Streptococcus epidemicus, S. pyogenes,* and *S. zymogenes* require riboflavin, as well as other growth substances. Rane & Subbarow (72) observed only slightly beneficial effects of flavin on any *Pneumococcus.* Snell & Strong (18) studied the effect of riboflavin on lactic acid bacteria on media very carefully freed of riboflavin. *Bacillus lactis-acidi, Lactobacillus casei, L. delbrükii,* and *L. gayoni* required riboflavin. *Lactobacillus arabinosus, L. pentosus, L. pentoaceticus, L. mannitopoeus, Bacillus brassicae, Streptococcus lactis* and *Leuconostoc mesenteroides* did not require riboflavin and were not benefited by its addition to the culture media. *Lactobacillus pentosus, B. brassicae, S. lactis,* and *Leuconostoc mesenteroides* produced relatively large amounts of riboflavin as measured by the growth of *Lactobacillus casei* and confirmed by a lumiflavin test. Synthetic riboflavin and riboflavin from natural sources were equally effective. The activity of twelve synthetic flavins was compared with that of riboflavin which is 6,7-dimethyl-9-(d-1'-ribityl)-isoalloxazine. Three closely related compounds, 6-ethyl-7-methyl-9-(d-1'-ribityl)-isoalloxazine, 7-methyl-9-(d-1'-ribityl)-isoalloxazine, and 6-methyl-9-(d-1'-ribityl)-isoalloxazine, permitted growth and lactic acid production through six passages without serious diminution of their effect but were from 10 to 60 per cent less effective than riboflavin for *L. casei* and *B. lactis acidi.* Four other compounds, 6,7-dimethyl-9-(d-1'-arabityl)-isoalloxazine, 6,7-dimethyl-9-(l-1'-arabityl)-isoalloxazine, 6-ethyl-7-methyl-9-(l-1'-arabityl)-isoalloxazine, and 5,6-benzo-9-(d-1'-ribityl)-isoalloxazine, were ineffective when used alone but were active in the presence of suboptimal quantities of riboflavin. The other five compounds, 6,7-dimethyl-9-(1'-sorbityl)-isoalloxazine, 9-(l-1'-arabityl)-isoalloxazine, 6,7,9-trimethyl-isoalloxazine (lumiflavin), 6,7-dimethyl-alloxazine (lumichrome), and riboflavin tetraacetate, were ineffective for bacteria although rats could use the latter. Riboflavin has not been found to be a growth substance for the yeasts or filamen-

tous fungi. Dennison (98) reported that eggplants in silica gravel with Withrow's nutrient solution grew much more rapidly than the controls when 2.5 P.P.M. of synthetic riboflavin were added.

Nicotinic acid.—Nicotinic acid was identified as a growth substance for *Staphylococcus aureus* by Knight (99) in 1937. It has been found to be important also for certain strains of diphtheria bacteria (100), dysentery bacilli (101), *Proteus* (102), *Brucella* (36), and *Salmonella paratyphi* A (103). Nicotinic acid shows considerable specificity for *Staphylococcus aureus* (44a), *Proteus vulgaris* (102), and for excised pea roots (104). Schmelkes (46) reported that thiazole-5-carboxylic acid, isosteric with nicotinic acid, stimulates dysentery bacilli. Nicotinic acid enters into the synthesis of some pyridine nucleotides such as Warburg's coenzyme (triphosphopyridine nucleotide) or cozymase (diphosphopyridine nucleotide) (105).

Addicott & Devirian (106) found nicotinic acid to be a growth substance for excised pea roots; Bonner & Devirian (39) and Bonner (40) reported that it was important for the growth of excised roots of radish, alfalfa, clover, cotton, *Datura,* and tomato, but not beneficial for flax or carrot.

Ascorbic acid.—Although it occurs abundantly in plants, evidence for deficiencies of ascorbic acid is slight. Kohman & Porter (107) reported that there was a rapid loss of ascorbic acid in tomato plants kept in the laboratory overnight and a rapid recovery when the plants were exposed to direct sunlight. Dennison (98) stated that ascorbic acid applied to tobacco plants in gravel cultures materially increased their growth. Cooper (108) reported that ascorbic acid favorably affected the germination of papaya pollen and the germination of the spores of *Colletotrichum gloeosporioides*. Bonner & Bonner (109) found embryos of some kinds of plants to respond favorably to ascorbic acid. The results of Rytz (55) were largely negative.

Amino acids.—Amino acids have long been regarded as important for the growth of many microorganisms, and protein hydrolysates have frequently been used as constituents of media. The greater availability of pure amino acids has encouraged investigation of the role of specific amino acids. For example Woolley & Hutchings (69) have reported for two species of hemolytic streptococci the simplest effective amino acid mixture to be composed of tryptophane, glutamic acid, isoleucine, lysine, arginine, tyrosine and cystine. Landy (110) found glutamine to be an essential growth substance for most strains of *Streptococcus haemolyticus*. The importance of β-alanine for yeast was recognized

by Williams & co-workers (43a) and for diphtheria bacteria by Mueller & Cohen (71a). Nielsen & Hartelius (111, 112) have carried on extensive investigations of amino acids as growth substances for yeast. White (113) considered glycine to be important for the growth of excised tomato roots although Robbins & Schmidt (21) and Bonner (40) secured vigorous growth in media which lacked glycine. Ellis & Spizizen (114) reported glycine to be an essential factor for the growth of bacteriophage. Bonner & Haagen-Smit (115) found purine bases, especially adenine, important for leaf growth. Although formerly regarded primarily as building stones for proteins, some amino acids are constituents of coenzymes or of growth substances; β-alanine forms a part of pantothenic acid and adenine is included in Warburg's coferment and in cozymase.

Unidentified growth substances.—There is a considerable number of growth substances for plants which have not been identified, and some probably represent additions to those now known. Hellinga (116) presented evidence for a substance produced by fungi which markedly affects the respiration of potato tuber tissue. Extracts containing 0.2 μg. dry matter were effective. The material was present also in yeast extracts and peptone. It is thermostable, adsorbed by charcoal and asbestos, eluted with ammoniacal acetone, nonvolatile, insoluble in ether and chloroform, and precipitated by barium hydroxide. It is not biotin, thiamin, lactoflavin, inositol, ascorbic acid, indole-3-acetic acid, glutathione, nicotinic acid, or histidine. Robbins & Hamner (77) have reported unidentified factors influencing the development of *Phycomyces,* and Schopfer & co-workers (117) have indicated that unidentified factors are present in wheat germ which favorably affect the growth of various Mucoraceae. Knight's "sporogenes vitamin," as well as Bios II B (118) and a number of other factors, is still unidentified.

SIMILARITIES IN GROWTH SUBSTANCE REQUIREMENTS

Although differences exist in the growth substance deficiencies of closely related organisms, even between strains of the same species, nevertheless certain deficiencies seem characteristic of some groups. For example, some of the lactic acid bacteria and some of the animal pathogens respond to riboflavin. There is little evidence, however, for a positive effect of this substance on other bacteria, yeasts, or the filamentous fungi. Pantothenic acid which has been found to be im-

portant for many bacteria and yeasts has not as yet been demonstrated to be a supplement for the filamentous fungi. The genus *Phytophthora* (119) as a group appears to require thiamin as such while the closely related genus *Pythium* (120) responds to thiamin, its intermediates, or pyrimidine alone.

Many organisms show deficiencies for some growth substances, for example, thiamin and nicotinic acid, while few have been demonstrated to lack adequate amounts of others, for example, pimelic acid. Further evidence that many growth substances (biotin, thiamin, riboflavin, panthothenic acid, *p*-amino benzoic acid) function for both plants and animals emphasizes the fundamental nature of these substances and adds to the interest of work with those important for plants.

LITERATURE CITED

1. Koser, S. A., and Saunders, F., *Bact. Rev.*, 2, 99 (1938)
2. Schopfer, W. H., *Ergeb. Biol.*, 16, 4 (1939)
3. Janke, A., *Zentr. Bakt. Parasitenk.*, *II*, 100, 409 (1939)
4. White, P. R., *Am. J. Botany*, 27, 811 (1940)
5. Steinberg, R. A., *J. Agr. Research*, 59, 749 (1939)
6. Robbins, W. J., and Kavanagh, F., *Bull. Torrey Botan. Club*, 65, 453 (1938)
7. Robbins, W. J., *Botan. Gaz.*, 101, 428 (1939)
8. Richardson, G. M., *Biochem. J.*, 30, 2184 (1936)
9. Fromageot, C., and Tchang, J. L., *Arch. Mikrobiol.*, 9, 434 (1938)
10. Hall, H. H., James, L. H., and Stuart, L. S., *Ind. Eng. Chem., Ind. Ed.*, 25, 1052 (1933)
11. Allison, F. E., *Proc. 3rd Intern. Congr. Microbiol.*, 486 (1939)
12. Allison, F. E., and Hoover, S. R., *J. Bact.*, 27, 561 (1934)
13. Robbins, W. J., and Schmidt, M. B., *Botan. Gaz.*, 99, 671 (1938)
14. Robbins, W. J., *Am. J. Botany*, 26, 772 (1939)
15. Schopfer, W. H., and Rytz, Jr., W., *Arch. Mikrobiol.*, 8, 244 (1937)
16. Koser, S. A., Chinn, B. D., and Saunders, F., *J. Bact.*, 36, 57 (1938)
17. Robbins, W. J., and Kavanagh, F., *Proc. Natl. Acad. Sci. U.S.*, 24, 141 (1938)
18. Snell, E. E., and Strong, F. M., *Enzymologia*, 6, 186 (1939)
19. Williams, R. J., and Saunders, D. H., *Biochem. J.*, 28, 1887 (1934)
20. Hawker, L. E., *Ann. Botany*, 3, 657 (1939)
21. Robbins, W. J., and Schmidt, M. B., *Am. J. Botany*, 26, 149 (1939)
22. Schopfer, W. H., *Arch. Mikrobiol.*, 9, 116 (1938)
23. Miller, W. L., *J. Chem. Education*, 7, 257 (1930)
24. Kögl, F., and Tönnis, B., *Z. physiol. Chem.*, 242, 43 (1936)
25. Schopfer, W. H., *Congr. microbiol. langue franç., 1st Congr.*, Paris (1938)
25a. Schopfer, W. H., *Ber. Schweiz. Botan. Ges.*, 47, 460 (1937)
26. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 62, 175 (1940)
27. Robbins, W. J., and Schmidt, M. B., *Bull. Torrey Botan. Club*, 66, 139 (1939)
28. Snell, E., Strong, F. M., and Peterson, W. H., *J. Bact.*, 38, 293 (1939)
29. Sinclair, H. M., *Biochem. J.*, 32, 2185 (1938)
30. Schopfer, W. J., and Jung, A., *Compt. rend. congr. intern. tech. chim. ind. agr., 5th Congr.*, Schéveningue, 1, 22 (1937)
31. Bonner, J., and Erickson, J., *Am. J. Botany*, 25, 685 (1938)
32. Bonner, J., and Buchman, E. R., *Proc. Natl. Acad. Sci. U.S.*, 25, 164 (1939)
33. Schultz, A. S., Atkin, L., and Frey, C. N., *J. Am. Chem. Soc.*, 61, 1931 (1939)
34. Tauber, H., *J. Chem. Education*, 16, 10 (1939)
35. Noecker, N. L., *Am. J. Botany*, 25, 345 (1938)
36. Kerby, G. P., *J. Bact.*, 37, 495 (1939)
37. Melin, E., and Lindeberg, G., *Botan. Notiser*, 241 (1939)

38. LINDEBERG, G., *Svensk Botan. Tid.,* **33,** 85 (1939)
39. BONNER, J., AND DEVIRIAN, P. S., *Am. J. Botany,* **26,** 661 (1939)
40. BONNER, J., *Am. J. Botany,* **27,** 692 (1940)
41. SCHOPFER, W. H., AND BLUMER, S., *Enzymologia,* **8,** 261 (1940)
42. SCHULTZ, A. S., ATKIN, L., AND FREY, C. N., *J. Bact.,* **40,** 339 (1940)
43. WILLIAMS, R. J., EAKIN, R. E., AND SNELL, E. E., *J. Am. Chem. Soc.,* **62,** 1204 (1940)
43a. WILLIAMS, R. J., AND ROHRMAN, E., *J. Am. Chem. Soc.,* **58,** 695 (1936)
44. BONNER, J., *Am. J. Botany,* **25,** 543 (1938)
44a. KNIGHT, B. C. J. G., AND MCILWAIN, H., *Biochem. J.,* **32,** 1241 (1938)
45. BLUMER, S., AND SCHOPFER, W. H., *Ber. Schweiz. Botan. Ges.,* **50,** 248 (1940)
46. SCHMELKES, F. C., *Science,* **90,** 113 (1939)
47. TRACY, A. H., AND ELDERFIELD, R. C., *Science,* **92,** 180 (1940)
48. BUCHMAN, E. R., AND RICHARDSON, E. M., *J. Am. Chem. Soc.,* **61,** 891 (1939)
49. BONNER, J., AND BUCHMAN, E. R., *Proc. Natl. Acad. Sci. U.S.,* **24,** 431 (1938)
50. ROBBINS, W. J., *Plant Physiol.,* **15,** 547 (1940)
51. ROBBINS, W. J., *Bull. Torrey Botan. Club.,* **67,** 565 (1940)
52. MCCLARY, J. E., *Proc. Natl. Acad. Sci. U.S.,* **26,** 581 (1940)
53. LILLY, V. G., AND LEONIAN, L. H., *Science,* **89,** 292 (1939)
54. BONNER, J., AND GREENE, J., *Botan. Gaz.,* **100,** 226 (1938)
55. RYTZ, JR., W., *Ber. Schweiz. Botan. Ges.,* **49,** 339 (1939)
56. BURKHOLDER, P. R., AND MCVEIGH, I., *Am. J. Botany,* **27,** 634 (1940)
57. BURKHOLDER, P. R., AND MCVEIGH, I., *Am. J. Botany,* **27,** 853 (1940)
58. BONNER, J., *Am. J. Botany,* **27,** 20s (1940)
59. BONNER, J., *Botan. Gaz.,* **101,** 491 (1939)
60. BONNER, D. M., AND BONNER, J., *Am. J. Botany,* **27,** 38 (1940)
61. ARNON, D. I., *Science,* **92,** 264 (1940)
62. HAMNER, C. L., *Botan. Gaz.,* **102,** 156 (1940)
63. TINCKER, M. A. H., *J. Roy. Hort. Soc.,* **64,** 554 (1939)
64. STILLER, E. T., HARRIS, S. A., FINKELSTEIN, J., KERESZTESY, J. C., AND FOLKERS, K., *J. Am. Chem. Soc.,* **62,** 1785 (1940)
65. JUKES, T. H., *J. Am. Chem. Soc.,* **61,** 975 (1939)
66. WOOLLEY, D. W., WAISMAN, H. A., AND ELVEHJEM, C. A., *J. Am. Chem. Soc.,* **61,** 977 (1939)
67. HOFFER, M., AND REICHSTEIN, T., *Nature,* **144,** 72 (1939)
68. MITCHELL, H. K., SNELL, E. E., AND WILLIAMS, R. J., *J. Am. Chem. Soc.,* **62,** 1791 (1940)
69. WOOLLEY, D. W., AND HUTCHINGS, B. L., *J. Bact.,* **39,** 287 (1940)
70. SUBBAROW, Y., AND RANE, L., *J. Am. Chem. Soc.,* **61,** 1616 (1939)
71. MUELLER, J. H., AND KLOTZ, A. W., *J. Am. Chem. Soc.,* **60,** 3086 (1938)
71a. MUELLER, J. H., AND COHEN, S., *J. Bact.,* **34,** 381 (1937)
72. RANE, L., AND SUBBAROW, Y., *J. Bact.,* **40,** 695 (1940)
73. MITCHELL, H. K., WEINSTOCK, H. H., SNELL, E. E., STANBERY, S. R., AND WILLIAMS, R. J., *J. Am. Chem. Soc.,* **62,** 1776 (1940)
74. RUBBO, S. D., AND GILLESPIE, J. M., *Nature,* **146,** 838 (1940)

75. Möller, E. F., *Z. physiol. Chem.*, **254**, 285 (1938)
76. Vilter, S. P., and Spies, T. D., *Science*, **91**, 200 (1940)
77. Robbins, W. J., and Hamner, K. C., *Botan. Gaz.*, **101**, 912 (1940)
78. Jung, A., and Schopfer, W. H., *Verh. Ver. Schweiz. Physiol.* (1939)
79. Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **61**, 1932 (1939)
80. Robbins, W. J., *Science*, **92**, 416 (1940)
81. Stoutemyer, V. T., *Am. Nurseryman*, **72**, 11, 32 (1940)
82. Kögl, F., and Fries, N., *Z. physiol. Chem.*, **249**, 93 (1937)
83. Fries, N., *Symb. botan. upsalienses*, **3**, 1 (1938)
84. Kögl, F., and van Wagtendonk, W. J., *Rec. trav. chim.*, **57**, 757 (1938)
85. West, P. M., and Wilson, P. W., *Enzymologia*, **8**, 152 (1940)
86. Allison, F. A., Hoover, S. R., and Burk, D., *Science*, **78**, 217 (1933)
87. György, P., Rose, C. S., Hoffman, K., Melville, D. B., and Vigneaud, V. du, *Science*, **92**, 609 (1940)
88. Moewus, F., *Jahrb. wiss. Botan.*, **86**, 753 (1938)
89. Raper, J. R., *Am. J. Botany*, **26**, 639 (1939)
90. Raper, J. R., *Am. J. Botany*, **27**, 162 (1940)
91. Mueller, J. H., *Science*, **85**, 502 (1937)
92. Hall, R. P., *Arch. Protistenk.*, **92**, 315 (1939)
93. English, J., Bonner, J., and Haagen-Smit, A. J., *J. Am. Chem. Soc.*, **61**, 3434 (1939)
94. van Overbeek, J., *Proc. Natl. Acad. Sci. U.S.*, **26**, 441 (1940)
95. Orla-Jensen, S., Otte, N. C., and Snog-Kjaer, A., *Zentr. Bakt. Parasitenk., II*, **94**, 434 (1936)
96. Rane, L., and Subbarow, Y., *Proc. Soc. Exptl. Biol. Med.*, **38**, 837 (1938)
97. Woolley, D. W., and Hutchings, B. L., *J. Bact.*, **38**, 285 (1939)
98. Dennison, R., *Science*, **92**, 17 (1940)
99. Knight, B. C. J. G., *Biochem. J.*, **31**, 966 (1937)
100. Mueller, J. H., *J. Bact.*, **34**, 429 (1937)
101. Koser, S. A., Dorfman, A., and Saunders, F., *Proc. Soc. Exptl. Biol. Med.*, **38**, 311 (1938)
102. Pelczar, M. J., and Porter, J. R., *J. Bact.*, **39**, 429 (1940)
103. Kligler, I. J., and Grosowitz, N., *J. Bact.*, **38**, 309 (1939)
104. Bonner, J., *Plant Physiol.*, **15**, 553 (1940)
105. Fildes, P., *Brit. J. Exptl. Path.*, **19**, 239 (1938)
106. Addicott, F. T., and Devirian, P. S., *Am. J. Botany*, **26**, 667 (1939)
107. Kohman, E. F., and Porter, D. R., *Science*, **92**, 561 (1940)
108. Cooper, W. C., *Botan. Gaz.*, **100**, 844 (1939)
109. Bonner, J., and Bonner, D. M., *Proc. Natl. Acad. Sci. U.S.*, **24**, 70 (1938)
110. Landy, M., *Nature*, **144**, 512 (1939)
111. Hartelius, V., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**, 323 (1939)
112. Nielsen, N., and Hartelius, V., *Compt. rend. trav. lab. Carlesberg, Sér. physiol.*, **22**, 375 (1939)
113. White, P. R., *Plant Physiol.*, **14**, 527 (1939)
114. Ellis, E. L., and Spizizen, J., *Science*, **92**, 91 (1940)
115. Bonner, D. M., and Haagen-Smit, A. J., *Proc. Natl. Acad. Sci. U.S.*, **25**, 184 (1939)

116. HELLINGA, J. J. A., *Proc. Acad. Sci. Amsterdam,* **43**, 249 (1940)
117. SCHOPFER, W. H., *Z. Vitaminforsch.,* **4**, 187 (1935)
118. MILLER, W. L., *Trans. Roy. Soc. Can., III,* **31**, 159 (1937)
119. ROBBINS, W. J., *Proc. Natl. Acad. Sci. U.S.,* **24**, 53 (1938)
120. ROBBINS, W. J., AND KAVANAGH, F., *Am. J. Botany,* **25**, 229 (1938)

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SPECTROMETRIC STUDIES IN RELATION TO BIOLOGY

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The absorption spectra of biologically active compounds have been the subject of extensive study and much information in this field has been accumulated during the past few years. For the most part, however, such data do not have any great bearing on biological problems except in the cases in which they may be used as an aid in the identification of purified biologically important compounds. With few exceptions the concentration of a compound in any organism is so small that it cannot be identified spectroscopically unless it is at least somewhat concentrated from the medium in which it exists. We do not regard it as appropriate to include in this review a list of all the recently determined absorption spectra that may be of biological interest; nor could we adequately describe such data without graphical representation. Rather we shall consider a small number of such cases only to illustrate the application of absorption spectroscopy to problems of compound identification. We shall confine our attention largely to problems in which either the prism or grating spectrophotometer has been used for analytical purposes in studying the mechanisms of certain chemical reactions occurring in biological systems. Such studies involve the blood pigments and particularly the respiratory enzymes.

We have restricted the subject matter because of lack of adequate space and because we wish to emphasize those aspects of this field which we believe will be of greater future importance. We also wish to present this review in such a form that it will be useful to those who wish to interpret spectroscopic data. Since this is the first review on this subject to appear in this series its content must necessarily include more than the work of the past year.

THE SPECTROMETRIC METHOD

The diminution in intensity, $-dI$, when monochromatic light passes through an infinitesimally thick layer, dl, of an absorbing medium is expressed by the equation

$$-dI = KIcdl \tag{1}$$

in which c is the concentration of the absorbing substance and I, the intensity of the light. In this equation K is a measure of light absorbing capacity of the substance in question.

Upon integration between the limits 0 to l (the length of the absorbing layer) the equation becomes

$$\ln_e \frac{I}{I_o} = -Kcl, \text{ or } \frac{I}{I_o} = e^{-Kcl}, \text{ or } \ln_e \frac{I_o}{I} = Kcl. \tag{2}$$

In these last equations the natural logarithms are used. I_o is the intensity of the incident, and I the intensity of the emergent light. When used with the natural logarithms, K is known as the molar absorption coefficient if c is expressed in moles per liter, and as the specific absorption coefficient, if the concentration is given in grams per liter. Some authors even express concentration as moles per cubic centimeter. In such a case the absorption coefficient so defined is 1,000 times the molar absorption coefficient.

If equation 2 is converted to common logarithms (i.e., to the base 10) it becomes

$$\log_{10} \frac{I_o}{I} = \frac{K}{2.303} cl, \tag{3}$$

or by letting $\frac{K}{2.303} = E$, $\log_{10} \frac{I_o}{I} = Ecl.$ $\tag{4}$

In equation 4, E is either the molar or specific extinction coefficient depending upon the units in which the concentration of the absorbing substance is expressed—in moles per liter or in grams per liter respectively.[1]

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[1] There has been no uniformity in the use of symbols to express the absorption and extinction coefficients and extinction coefficient and absorption coefficient are often interchanged. The reader should always ascertain the particular definition the author is using. An attempt to standardize nomenclature in this field is being made through a committee of the National Research Council.

When E and c are combined into one constant so that $Ec = X$ then

$$\log_{10} \frac{I_o}{I} = Xl. \tag{5}$$

X is then called merely the extinction coefficient. The extinction coefficient per unit of cell length is known as the extinction or optical density; that is, the extinction or optical density is equal to $\dfrac{\log_{10} \dfrac{I_o}{I}}{l}$ or merely equal to $\log_{10} \dfrac{I_o}{I}$ when the cell length is equal to one centimeter. The same considerations apply to the absorption coefficients, with the exception that absorption is interchanged with extinction.

It is common European practice to use absorption coefficients while the American practice leans more toward the use of logarithms to the base 10 and the consequent extinction coefficients.

Equations 1 to 4 are all expressions of the familiar Beer-Lambert law. The validity of this law is often questioned but in all dilute solutions of highly colored substances such as the blood pigments it is valid (1 to 5).

The values of the extinction coefficients vary with the wave length. A plot of the molar extinction coefficients against wave length (or wave number) constitutes an absorption spectrum.

The extinction coefficient for a designated solute may be obtained by subtracting the extinction due to all other substances in solution from the extinction due to these same substances plus that solute. The total extinction for all substances is

$$\log \frac{I_o}{I} = (E_1 C_1 + \Sigma E_i C_i)l. \tag{6}$$

The subscript 1 refers to the designated solute and $\Sigma E_i C_i$ represents the sum of the extinction coefficients for all substances other than that solute. When the designated solute is not present

$$\log \frac{I_o}{I'} = (\Sigma E_i C_i)l. \tag{7}$$

Subtracting 7 from 6

$$\log \frac{I'}{I} = E_1 C_1 l. \tag{8}$$

The value of the molar extinction coefficient of the specific solute, E_1, is obtained automatically if one determines the light intensity, I, passing through the solution containing the solute in question and the light intensity I' passing through the solution containing all other constituents except that solute. E_1 is then calculated from formula 8. By this procedure corrections for scattered light and reflection are also made automatically.

If the molar extinction coefficient of a particular substance for a particular wave length is known then it is obvious (from equation 8) that the concentration of this substance may be determined merely by obtaining the experimental values I and I'. This is the general principle employed in analytical absorption spectroscopy.

The error incurred in determining either the extinction coefficient or the concentration will depend upon the relative magnitudes of I and I'. It can be shown (4) that the per cent error in determining Ec (equation 8) is a minimum when $\log \dfrac{I'}{I} = 0.4343$, or when $\dfrac{I'}{I} = 2.72$.

The concentrations of two (or more) solutes in the same solution which have different absorption spectra may be determined by employing two different and suitable wave lengths. Under such circumstances

$$\log \frac{I_1'}{I_1} = (E_{1A}C_A + E_{1B}C_B)l \tag{9}$$

$$\text{and } \log \frac{I_2'}{I_2} = (E_{2A}C_A + E_{2B}C_B)l. \tag{10}$$

E_{1A} and E_{1B} are the molar extinction coefficients of substances A and B respectively for wave length 1 and E_{2A} and E_{2B} are these same quantities for wave length 2. With E_{1A}, E_{2A}, E_{1B}, and E_{2B} known and with I_1, I_1', I_2, and I_2' determined directly, the only unknown quantities are C_A and C_B. The values of both C_A and C_B can be obtained by simultaneous solutions of equations 9 and 10. The different "methods" of simultaneously determining two constituents only involve different combinations of equations 9 and 10, or the use of them in a restricted way. Thus in the method of Vierordt (6) equations 9 and 10 are combined in such a way that

$$C_A = \frac{E_{2B} \log \dfrac{I_2'}{I_2} - E_{1B} \log \dfrac{I_1'}{I_1}}{E_{1A}E_{2B} - E_{2A}E_{1B}} \tag{11}$$

$$\text{and } C_B = \frac{E_{2A} \log \dfrac{I_1'}{I_1} - E_{1A} \log \dfrac{I_2'}{I_2}}{E_{2A}E_{1B} - E_{1A}E_{2B}}. \tag{12}$$

In the method of Hufner (7) these equations are combined so that the per cent composition of each of the unknown substances is determined directly from the formula.

If the total concentration is known (as is often the case when dealing with oxygenated and unoxygenated hemoglobin) the determination is simplified and a determination at one wave length only is necessary to obtain the required information. If C_t is total concentration then

$$\log \frac{I'}{I} = E_{1A}C_A + E_{1B}(C_t - C_A). \tag{13}$$

Since C_A is the only unknown, one equation only is necessary for a solution of its value. A convenient method of determining C_t, the total concentration, is that of selecting a wave length for which both substances have the same molar extinction coefficients. Then at this wave length

$$\log \frac{I'}{I} = E_{AB}C_t l. \tag{14}$$

The principles discussed here may be extended to the analysis of three or more components in the same solution. In each case as many different wave lengths must be employed as there are separate constituents included in the analysis.

Table I will be found convenient in converting one kind of absorption or extinction coefficient into another.

Spectrophotometric Methods

The various methods of obtaining spectrographic or spectrophotometric data have been adequately reviewed (5, 8, 9) so that it is only necessary to outline these in the briefest possible way. Three methods are in general use for getting spectrographic data: (a) photographic, (b) visual, and (c) by the use of the photocell. The first of these when combined with a device such as the Spekker photometer is a convenient method for obtaining spectra of relatively pure compounds for the purpose of identification. For analytical purposes, it is not convenient, nor is it very accurate, except in the hands of an expert. The second method, that of visual observation, is not an objective one and is limited to the visual range within the spectrum. The use of

TABLE I
CONVERSION FACTORS* FOR ABSORPTION AND EXTINCTION COEFFICIENTS†

	Absorption Coefficients‡ (involving logarithms to base e)				Extinction Coefficients‡ (involving logarithms to base 10)			
	$\dfrac{\text{liters}}{\text{moles} \times \text{cm.}}$	$\dfrac{\text{cc.}}{\text{moles} \times \text{cm.}}$	$\dfrac{\text{liters}}{\text{gm.} \times \text{cm.}}$	$\dfrac{\text{cc.}}{\text{gm.} \times \text{cm.}}$	$\dfrac{\text{liters}}{\text{moles} \times \text{cm.}}$	$\dfrac{\text{cc.}}{\text{moles} \times \text{cm.}}$	$\dfrac{\text{liters}}{\text{gm.} \times \text{cm.}}$	$\dfrac{\text{cc.}}{\text{gm.} \times \text{cm.}}$
Absorption Coefficients $\dfrac{\text{liters}}{\text{moles} \times \text{cm.}}$	1	.001	M§	.001 M	2.303	2.303×10^{-3}	2.303 M	2.303×10^{-3} M
$\dfrac{\text{cc.}}{\text{moles} \times \text{cm.}}$	1000	1	1000 M	M	2303	2.303	2303 M	2.303 M
$\dfrac{\text{liters}}{\text{gm.} \times \text{cm.}}$	$\dfrac{1}{M}$	$\dfrac{.001}{M}$	1	.001	$\dfrac{2.303}{M}$	$\dfrac{2.303 \times 10^{-3}}{M}$	2.303	2.303×10^{-3}
$\dfrac{\text{cc.}}{\text{gm.} \times \text{cm.}}$	$\dfrac{1000}{M}$	$\dfrac{1}{M}$	1000	1	$\dfrac{2303}{M}$	$\dfrac{2.303}{M}$	2303	2.303
Extinction Coefficients $\dfrac{\text{liters}}{\text{moles} \times \text{cm.}}$	0.434	0.434×10^{-3}	0.434 M	0.434×10^{-3} M	1	.001	M	.001 M
$\dfrac{\text{cc.}}{\text{moles} \times \text{cm.}}$	434	0.434	434 M	0.434 M	1000	1	1000 M	M
$\dfrac{\text{liters}}{\text{gm.} \times \text{cm.}}$	$\dfrac{0.434}{M}$	$\dfrac{0.434 \times 10^{-3}}{M}$	0.434	0.434×10^{-3}	$\dfrac{1}{M}$	$\dfrac{.001}{M}$	1	.001
$\dfrac{\text{cc.}}{\text{gm.} \times \text{cm.}}$	$\dfrac{434}{M}$	$\dfrac{0.434}{M}$	434	0.434	$\dfrac{1000}{M}$	$\dfrac{1}{M}$	1000	1

* To convert values of coefficients designated by the units in the top row into values designated by units in the first vertical column, multiply by the factor in the corresponding space.
† Coefficients indicated by units.
‡ The terms "absorption" and "extinction" have often been used interchangeably.
§ M refers to molecular weight of solute.

the photoelectric method is rapidly being developed and promises to be much more widely used.

In general the spectrophotometric apparatus necessary for the obtaining of spectral data consists of a continuous light source, a monochromator to resolve the light into a spectral distribution, the absorption tube, a photoelectric cell, and an electrical instrument or vacuum-tube circuit for measuring the photoelectric current. The European biological laboratories, including those of O. Warburg, H. Theorell, H. v. Euler, and Richard Kuhn, to a large extent have adopted the photoelectric method which employs a double monochromator to obtain purer light, and a string electrometer for measuring the photoelectric current (10).

Hogness, Zscheile & Sidwell (4) have described in detail an arrangement whereby a single monochromator of high optical quality is used instead of the double monochromator. The photoelectric current is amplified by an electrometer vacuum tube. Installations of this kind, with some variations, are now in operation in the Department of Agriculture, Beltsville, Maryland; the University of Minnesota; Purdue University; and the University of Pennsylvania. The Purdue installation employs the Hilger-Müller double monochromator.

A similar installation employing a circuit with two matched electrometer tubes is in operation at the Phillips Lamps Works of Eindhoven, Holland (11).

Very recently similar installations limited to 3200 Å units in the ultraviolet region embodied in one assembled unit have appeared on the market. Such apparatus, because of their greater utility, will without doubt replace the colorimeter.

The culmination of the efforts along these lines appears in the high-speed recording spectrophotometer designed by Harrison (12, 13).

IDENTIFICATION OF UNKNOWN SUBSTANCES

The judicious use of absorption spectroscopy can be of great assistance in the determination of the structure and in the identification of biologically active compounds. However, it should be emphasized that this field has not progressed to the point where it is possible to get a very clear insight into the organic structure from absorption spectra alone. These data must be combined with chemical data before full advantage can be taken of them.

The "color" of a compound, i.e., the characteristic absorption of light, resides in a chromophoric group. If this chromophoric group is

not a free radical and if it consists only of carbon, hydrogen, oxygen, and nitrogen atoms, it is made up essentially of conjugated double bonds, and may include certain ring structures.

The effect of conjugation on the position of maximum absorption and on the molar absorption or extinction coefficients has been rather extensively studied by Hausser (14), Kuhn (15), and their co-workers. These investigators have shown that the absorption coefficient of the peak of the absorption band is a linear function of the number of double bonds. They also have shown that the absorption maximum is shifted toward longer wave lengths as the number of conjugated double bonds increases. In making these correlations they have found that the carbon-oxygen linkage in both carboxyl and aldehyde groups when conjugated with a carbon-carbon double bond behaves as a normal double bond while a phenyl group so conjugated has the effect of only one and one-half double bonds. The work in this field has been more recently extended and considered from a theoretical point of view by a number of investigators, particularly by Mulliken (16).

The rules of Hausser can often be useful in determining whether an absorption band of an unknown substance is compatible with an assumed structure. Whenever possible, the best method is to compare directly the absorption band of the unknown with that of a known substance having the same chromophoric structure as that assumed. Thus Warburg & Christian (17) found that the absorption spectrum of triphosphopyridine nucleotide (cozymase II) was almost identical with that of the trigonelline and with this information they showed that triphosphopyridine nucleotide was a derivative of the amide of nicotinic acid. Likewise in the determination of the structure of lumiflavin, which in turn led to the structure of riboflavin, Stern & Holiday (18) found the spectrographic data indispensable. The fact that andrenosterone has an absorption band with a maximum at about 240 mμ, led Reichstein (19) to conclude that its structure contained an α-β-unsaturated ketone. This same relationship between structure and absorption is apparent for androstenedione and cholestenone (20).

QUANTITATIVE ESTIMATIONS

Colorimetric analysis.—Ashley (5) has recently pointed out some of the advantages of spectrophotometric methods in the field of colorimetric analysis. Various colorimetric methods were compared on the basis of molecular extinction coefficients, since for a given change in concentration, the change in optical density will be greatest for the

greatest molar extinction coefficient. In the case of copper, for example, the molar extinction coefficients for the dithizonate and the diethyldithiocarbamate were 21,800 and 531 moles^{-1} $\times$ l. $\times$ cm.$^{-1}$ respectively, while other methods gave lower coefficients. Thus the relative sensitivity of different methods can be quantitatively expressed in terms of molar extinction coefficients. The majority of colorimetric methods involve colors which are the result of single broad absorption bands, and in these cases the various types of photoelectric colorimeters using filters are probably almost as good as devices which use monochromatic light. However, the use of the latter introduces the possibility of carrying out colorimetric analyses in the presence of other colored reagents, and consequently preliminary separation from colored materials is often unnecessary. In some cases it can be shown that the colored complex has its absorption band in a region in which the accompanying color transmits completely, as in the case of nickel determinations in the presence of iron (21). Even when this is not the case, it is possible to correct for the absorption of the interfering substances. By making extinction measurements at as many different wave lengths as there are constituents in the mixture, the concentration of any constituent can be calculated by the method previously indicated. The possibilities have been but slightly explored as yet, but there are numerous cases such as in the dithizone system (22) where the technique should eliminate the necessity for preliminary separation. Good examples of the spectrophotometric method of determining two constituents are the work of Zscheile (2) on chlorophylls *a* and *b* and the work of Miller *et al.* (23) on the determination of α- and β-carotene in the same solution. Miller has also made an analytical study of the ternary system, β-carotene, leaf xanthophyll, and lycopene (24, 25), and of the quaternary system, chlorophylls *a* and *b*, β-carotene, and leaf xanthophyll (26).

Blood pigments.—The application of spectrophotometric methods to the study of the blood pigments has been reviewed recently by Michel & Harris (27) who in addition made experimental application of the principles discussed and correlated the data available in the literature. Their work as well as a large amount of that in the existing literature was done with visual type spectrophotometers. Work of this nature can now be greatly facilitated by the use of photoelectric spectrophotometers, and at least two such instruments are commercially available at present (28). Since the total extinction coefficient for a solution is an additive function of the separate extinction coefficients of its constituents in the absence of interaction, the extinction

coefficient of a solute can be calculated by subtraction of the extinction of the remainder of the materials in solution from the total observed extinction (equations 6, 7, and 8). Michel & Harris have determined methemoglobin in oxyhemoglobin solutions according to the principle that if the composition of one colored component in a mixture can be altered without changing any of the other components, the concentration of that substance may be determined by measuring the extinction coefficient of the mixture before and after the alteration. In this case cyanide is added to convert methemoglobin to cyanmethemoglobin and the extinction is measured at 634 mμ before and after the cyanide addition. The change in extinction divided by a constant gives the concentration of methemoglobin. Cyanide does not change the extinction value of either oxyhemoglobin or sulfhemoglobin. Oxyhemoglobin may be converted to methemoglobin by oxidation with potassium ferricyanide, after which the addition of cyanide will give an amount of cyanmethemoglobin equal to the sum of the oxyhemoglobin and methemoglobin originally present. Sulfhemoglobin may be determined on the basis of the ratio of the extinction at 540 mμ to the extinction at 620 mμ, following the principles previously discussed. If methemoglobin is present it may be determined separately and these data must be incorporated into the calculation. Similar methods for these three blood pigments have been presented by Evelyn & Malloy (29) for use with the Evelyn colorimeter.

In addition to quantitative estimations, photoelectric spectrophotometry is admirably suited for the study of the physicochemical properties of hemoglobin and related compounds. A study of the reaction of ferriheme hydroxide with cyanide led to the conclusion that ferriheme exists in solution as a polymerized ferriheme hydroxide (hematin) and that the cyanide compound is unpolymerized (30). The absorption spectra of the two compounds from 2200 to 7000 Å were also given. In a study of the effect of electrolytes on the hemoglobin-oxygen equilibrium (31) it was shown that solutions of various salts inhibit the oxygenation of hemoglobin in proportion to their ionic strengths. It was further suggested that in some cases the anions combine with hemoglobin. The spectra of hemoglobin and oxyhemoglobin from 2200 to 7700 Å were also given. In all previous photometric investigations of hemoglobin and oxyhemoglobin the absorption bands in the spectral region between 540 and 580 mμ were used. In this work it was shown that a new band at 755 mμ was most satisfactory for the determination of oxyhemoglobin. On the assumption that the percentage oxygenation of a hemoglobin solution at a given

oxygen pressure, as determined spectrophotometrically, can be used as a criterion of purity, various methods of purification were studied (32), and adsorption on γ-aluminum hydroxide was found to give the best results. The purified hemoglobin was then used for the study of the hemoglobin-oxygen equilibrium (33) as affected by certain conditions, especially pH, temperature, and salts.

A spectrophotometric method for the analysis of the reduced and oxidized forms of two colored substances in equilibrium was described by Stotz, Sidwell & Hogness (34) and was used to determine the oxidation-reduction potential of pure cytochrome-c, for which E_0' was found to be $+0.262$ volt between pH 5.0 and 8.0. Altschul & Hogness (35) presented evidence for the formation of carboxycytochrome-c from reduced cytochome-c and carbon monoxide throughout the entire pH range from 3.8 to 13.0. Keilin had previously reported complex formation only in the alkaline range and Keilin & Hartree (36) have since questioned the position of Altschul & Hogness although the data are in essential agreement. Potter (37) has since presented evidence for the formation of cyanferricytochrome-c.

Hydrogen carriers.—This term is used to include the pyridine nucleotide coenzymes, cytochromes, and flavoproteins, which are discussed in greater detail in the section on respiratory enzymes. Obviously the quantitative spectrophotometric determination of the carriers presents no problem if the respective compounds are obtained in pure form. In these cases the concentration may be determined simply on the basis of the molecular extinction coefficients (38, 39), care being taken to have the compounds completely oxidized or reduced. The problem from an analytical standpoint is the concentration of the compound and if possible its determination in the presence of impurities, since the separation of impurities can seldom be achieved without entailing unknown losses of the compound in question.

Cytochrome-c has been determined in extracts containing other pigments by means of absorption measurements at several wave lengths. On the basis of the predetermined extinction coefficients of cytochrome and the principal impurities at the various wave lengths, it was possible to correct for the various impurities and thus calculate the cytochrome-c concentration (40). Perhaps a more satisfactory approach will be the specific enzymatic oxidation and reduction of the compound in question, with the concentration being calculated on the basis of the extinction between the oxidized and the reduced states and the difference in the molecular extinction coefficients for the oxidized and reduced states, as has been suggested in the case of cyto-

chrome-c (37). The same approach should be applicable to all the carriers.

Enzyme Studies Employing Spectrophotometric Methods

One of the outstanding developments in recent years has been the application of spectrophotometric methods to the study of the respiratory enzymes. Since all of the known compounds which function as hydrogen carriers exhibit changes in absorption, because of changes in conjugation when reduced or oxidized, it is possible to resolve the oxidation of most metabolites into various steps by means of the spectrophotometric observation of the oxidation or reduction of the specific carriers involved. The reactions listed below represent the steps in the oxidation of a model substrate (41):

Metabolite + pyridine nucleotide coenzyme (Co) $\rightleftharpoons$ Oxidized
    metabolite + reduced pyridine nucleotide coenzyme (CoH$_2$)   (I)

CoH$_2$ + flavin enzyme (F) $\rightleftharpoons$ Co + reduced flavin
    enzyme (FH$_2$)   (II)

FH$_2$ + 2 oxidized cytochrome-c (Fe^{+++}) $\rightleftharpoons$ F + 2 reduced
    cytochrome-c (Fe^{++}) + 2 H$^+$ (III)

$$2 \text{ Fe}^{++} + 2 \text{ H}^+ + \tfrac{1}{2} \text{ O}_2 \xrightarrow{\text{(oxidase)}} 2 \text{ Fe}^{+++} + \text{H}_2\text{O} \qquad \text{(IV)}$$

$$\text{Metabolite} + \tfrac{1}{2} \text{ O}_2 \longrightarrow \text{oxidized metabolite} + \text{H}_2\text{O} \qquad \text{(V)}$$
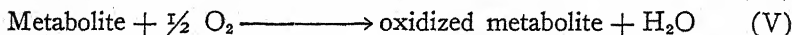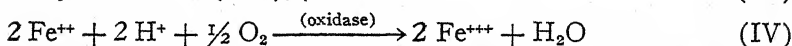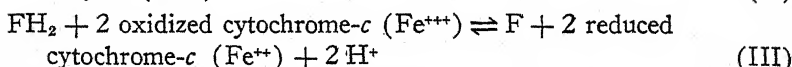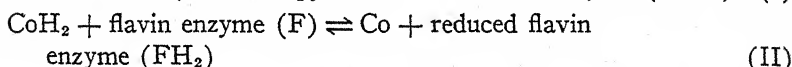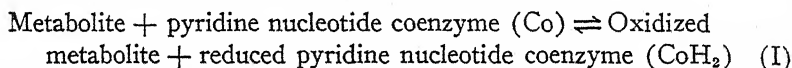
The over-all reaction, V, which represents the sum of the first four reactions, is readily measured in the familiar Warburg manometric apparatus, but it is generally difficult to study the partial reactions by this approach. It is at this point that the spectrophotometric technique demonstrates its usefulness, since the coenzymes, flavoproteins, and cytochromes all show characteristic changes in absorption when oxidized or reduced. It is therefore possible to gain further insight into the mechanism of the oxidation of the various metabolites as well as to study the exact locus of action of various factors such as pH, buffer, and substrate concentration, as well as the effect of various inhibitors and drugs.

Pyridine nucleotide coenzymes.—The application of photoelectric spectrophotometry to reactions involving the pyridine nucleotide coenzymes followed very rapidly after the announcement in 1935 by Warburg, Christian & Griese (42) that triphosphopyridine nucleotide (coenzyme II) contains a pyridine group which when reversibly reduced by two atoms of hydrogen exhibits a broad absorption band with a maximum at 340 mμ. It was soon shown by Euler, Adler &

Hellstrom (43) and by Warburg & Christian (44) that diphospho-pyridine nucleotide (coenzyme I) possesses an almost identical absorption band in the reduced state. The oxidized coenzymes show virtually no absorption at 340 mμ.

It has been abundantly demonstrated that the coenzymes function as carriers by virtue of the fact that they may be reversibly reduced by a single system, i.e., reaction I, which is catalyzed by various dehydrogenases, and that they may be reversibly oxidized by another system, i.e., reaction II, which involves a flavoprotein (41). Reaction I may be studied on the basis of the change in $\log I_0/I$ at 340 mμ provided that reaction II is not taking place simultaneously. Dialyzed aqueous extracts from acetone powders prepared from aqueous tissue extracts (45) do not catalyze reaction II, or at any rate do not catalyze the sum of reactions II, III, and IV, and appear to contain all of the coenzyme-linked dehydrogenases of the original tissue. Since the preparations are also free of substrates, it is possible to study any particular dehydrogenase by using the appropriate substrate. By this technique it has been shown that reaction I is an equilibrium reaction. For example, the addition of a small amount of alcohol to a solution containing enzyme and diphosphopyridine nucleotide results in a rise in the value for ($\log I_0/I$) at 340 mμ and either this value or its equivalent in moles of substrate or coenzyme may be plotted against time. When the reaction has come to a steady state, the addition of an excess of acetaldehyde results in a fall in extinction, due to the fact that the equilibrium position in reaction I is shifted to the left (43, 44). On the basis of this reaction the presence of dehydrogenases for lactate, glyceraldehyde phosphate, and α-phosphoglycerol have been demonstrated in muscle extracts (46), and numerous other similar applications have been made.

In a preparation containing several dehydrogenases it is possible to show the reduction of one substrate by the oxidation of another. Thus α-phosphoglycerol effected the reduction of diphosphopyridine nucleotide, and the subsequent addition of pyruvate resulted in the oxidation of the coenzyme (47). That the dismutation in the case of alcoholic fermentation is in reality due to the action of two distinct dehydrogenases has been demonstrated by Warburg & Christian (48) who used an initial reaction mixture containing diphosphopyridine nucleotide, glyceraldehyde phosphate, and acetaldehyde. Addition of pure glyceraldehyde phosphate dehydrogenase resulted in the reduction of the diphosphopyridine nucleotide as shown by the increase in the extinction at 340 mμ. The subsequent addition of pure alcohol

dehydrogenase resulted in the oxidation of the reduced coenzyme due to the catalysis of the reaction between the reduced coenzyme and acetaldehyde. A special case is that in which the oxidized product of one reaction is the reduced product of another. Thus in a crude preparation containing α-phosphoglycerol dehydrogenase and glyceraldehyde phosphate dehydrogenase it is not possible to study the dehydrogenation of glyceraldehyde phosphate for, although the latter is converted to phosphoglyceric acid, the observation of the reduction of diphosphopyridine nucleotide will be obscured by the fact that hydrogen from the reduced form will tend to be transferred to a part of the glyceraldehyde phosphate, converting it to α-phosphoglycerol, and the increase in the extinction will be partially cancelled out. Adler & Günther (49) overcame this difficulty by using a brain enzyme preparation which was rich in triose phosphate dehydrogenase and virtually lacking in α-phosphoglycerol dehydrogenase.

The quantitative estimation of the dehydrogenases in tissue extracts can be made on the basis of their activity in promoting reaction I, measuring the reaction rate in either direction spectrophotometrically. Thus Euler, Adler, Günther & Das (50) approximated the glutaric dehydrogenase content of tissue extracts on the basis of the reverse reaction, measuring the initial rate of oxidation of reduced diphosphopyridine nucleotide in the presence of α-ketoglutaric acid and ammonia. Euler, Adler & Günther (51) have measured the rate of the forward reaction in the case of the lactic and malic dehydrogenases, again obtaining values on the basis of the initial velocity. Since the initial reaction velocity is a somewhat uncertain quantity, it would seem that a more desirable method would be to determine the reaction order and the velocity constant in order to determine the enzyme concentration.

No satisfactory method for determining the enzyme content of various tissues in normal and abnormal states has thus far been devised and hence all distribution studies are only approximations at best. It has always been the practice to effect a partial purification of the enzyme before the estimation and since the per cent yield is an unknown quantity and may vary from tissue to tissue the data are always open to question. There is reason to believe (52) that the spectrophotometric approach may make possible the measurement of specific reactions in unpurified tissue preparations although this has not as yet been done.

The availability of a method for the quantitative estimation of dehydrogenase activity makes possible the study of the effect of

various environmental factors using the same methods. A few applications have been made including the inhibition of hexose monophosphate dehyrogenase (*Zwischenferment*) by phosphate (53), the inhibitory effect of iodoacetic acid on a number of dehydrogenases (54), and the activation of isocitric dehydrogenase by manganese and magnesium (55).

The spectrophotometric method has been particularly useful in determining the coenzyme specificity of various dehydrogenases, since the identity of the reduced coenzyme can be proved by showing reoxidation with a known system such as pyruvate-lactate (55).

In addition to the study of the dehydrogenase, reaction I, it is possible to study the enzymes responsible for the oxidation of the reduced coenzymes, i.e., reaction II, on the basis of the decrease in the extinction at 340 mμ. For this work it is necessary to prepare the reduced coenzymes chemically (56, 57) and to work in the absence of any hydrogen-donating system which might bring about reaction I. Thus far, all of the enzymes which oxidize the reduced coenzymes have been shown to be flavoproteins.[2]

Although it appears likely that the physiological oxidant of the yellow enzymes is cytochrome-*c* (52), oxygen and methylene blue may also be used (58). Studies on diaphorase I (59, 60, 61) and diaphorase II (56) have been made on the basis of the oxidation of the chemically reduced coenzymes using small amounts of methylene blue as an intermediary carrier and oxygen as the end acceptor (62). Diaphorase catalyzes the reaction between reduced diphosphopyridine nucleotide and methylene blue. The "old" yellow enzyme of Warburg & Christian reacts directly with oxygen; it is not necessary to add methylene blue as is the case with diaphorase or with the "new" yellow enzyme, in order to oxidize the reduced coenzymes (63). Fresh preparations of crude diaphorase will oxidize reduced diphosphopyridine nucleotide in the absence of methylene blue and the reaction is inhibited by dilute cyanide (62, 64), suggesting the participation of cytochrome oxidase. It has been shown that additions of cytochrome-*c* stimulate the rate of diphosphopyridine nucleotide oxidation in such preparations (64).

The flavoproteins.—The known yellow enzymes, i.e., the "old" (66) and "new" (67) yellow enzymes, amino acid oxidase (68), xanthine oxidase (69), cytochrome-*c* reductase (52), and the enzymes reported by Straub (70), and by Corran & Green (71), are charac-

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[2] With the exception of the dehydrogenases, which of course reduce the coenzymes reversibly (41).

terized by similar absorption spectra with absorption maxima at approximately 275, 385, and 455 mμ. Some of these contain the alloxazine mononucleotide and some the dinucleotide as coenzymes, nevertheless the chromophore, which is the alloxazine grouping, is the same and the spectra are therefore alike. These spectra have been used extensively in the chemical characterization and for quantitative determination of these compounds, but they have not been employed for the study of reactivity. Nor can the spectra of the flavoproteins be used to determine their own activity inasmuch as they are so active that with concentrations necessary for spectrophotometric measurements the reaction velocities are too great. With the exception of cytochrome-c reductase, either manometric methods or the methylene blue techniques have been used in testing for activity. With the reductase (52) an indirect method was employed which involved the spectrophotometric determination of the rate of reduction of cytochrome-c. This method permitted the use of very much smaller quantities of reductase than could have been used for the direct spectrophotometric determination of the reductase itself.

Cytochrome-c.—The sharp absorption band with a maximum at 550 mμ which is exhibited by reduced cytochrome-c together with the fact that this compound can be isolated in pure form (72) make it particularly suitable for the study of both reaction III and IV as well as the sum of I, II, and III, or of II and III. Since the oxidized form of the compound has an appreciable absorption at 550 mμ it is necessary to know the extinction coefficients for both forms. These data have been made available by Theorell (73). Considerable information has been derived from the pioneer work of Keilin (74) who in 1925 studied the appearance and disappearance of the reduced bands of the cytochromes *in situ* by means of visual spectroscopic observation.

The reactions leading to the reduction of cytochrome-c can be studied spectrophotometrically at the wave length 550 mμ if the reoxidation of cytochrome-c is prevented. This may be done by working in the absence of cytochrome oxidase (52), by working anaerobically (75) or by blocking the oxidase with cyanide (37, 75). The latter approach is complicated by the fact that cytochrome-c reacts with cyanide to form a compound which is not enzymatically reducible (37). The reduction of cytochrome-c by reduced diphosphopyridine nucleotide and by succinate (37, 64) as well as by the over-all system of hexose monophosphate, dehydrogenase, triphosphopyridine nucleotide, and flavoprotein has been studied (52). It has been found pos-

sible to study the oxidation of cytochrome-*c* by cytochrome oxidase by preparing chemically reduced cytochrome-*c* (76, 77).

Ball (78) has studied cytochromes *a*, *b*, and *c*, in crude preparations and has determined spectroscopically the ratio of oxidized to reduced forms in the presence of reversible systems of known potential. From this he was able to calculate the oxidation-reduction potential for all three cytochromes, and his value for cytochrome-*c* checks closely with determinations made by other means on pure cytochrome-*c* (34).

Urban & Peugnet (65) have attempted to follow the oxidation and reduction of cytochrome-*c* in muscle tissue *in situ*, using a spectrophotometric method similar to that employed by Millikan (79) for the study of muscle hemoglobin *in situ*.

Isolation of enzymes.—The concentration of an enzyme can be determined spectrophotometrically in two different ways: (*a*) the absorption, characteristic of the substance in question, can be determined directly and its concentration calculated by the use of equation 8; and (*b*) the concentration of any component of an enzyme system can be determined by the measurement of the velocity of oxidation or reduction of some other component of the system. The first of these methods is an obvious one. It suffers the disadvantage that relatively large quantities of the enzyme are necessary for each determination. The other method has been employed in the isolation and purification of cytochrome-*c* peroxidase (77) and cytochrome-*c* reductase (52). With the reductase the enzyme system employed involved the use of cytochrome-*c*, the reductase, triphosphopyridine nucleotide, "*Zwischenferment,*" and hexose monophosphate. The hexose monophosphate reduced the triphosphopyridine nucleotide; this in turn reduced the cytochrome reductase and the reductase reduced the cytochrome. The conditions were such that the rate of reduction of the cytochrome-*c* as measured spectrophotometrically was determined by the concentration of the reductase. In this way accurate assays of as little as 0.4 µg. of reductase could be made. By suitably changing the concentrations of the components this system can be employed to determine the concentration of any of its components. Cytochrome-*c* is to this method what oxygen is to the usual manometric procedure.

In a like manner cytochrome-*c* peroxidase was assayed by the use of reduced cytochrome-*c* which is oxidized by hydrogen peroxide in the presence of the peroxidase. The test can be used to accurately determine as little as a few hundredths of a microgram of the peroxi-

dase. This system may also be used to determine microquantities of hydrogen peroxide.

These are the only cases known to the authors for which spectrophotometric tests have been consistently used for purposes of enzyme isolation. The method seems to have great promise.

KINETICS

In spite of its promise not a great deal has yet been done in following spectroscopically the kinetics of enzyme reactions. The outstanding researches to date are probably those of Negelein & Wulff (80), and Negelein & Haas (53). The former work was concerned with the enzymatic reaction between alcohol and diphosphopyridine nucleotide producing acetaldehyde and dihydrodiphosphopyridine nucleotide. In this work the concentration of dihydrodiphosphopyridine nucleotide was determined from the magnitudes of the absorption band at 340 mμ. Knowing the concentration of this constituent at any given time and the initial concentrations, the concentrations of both of the reactants and the products could be calculated. These kinetic studies showed conclusively that all the reactants and products formed association products with the protein which acted as the catalyst. Their data allowed these investigators to calculate the dissociation constants of the protein-substrate and protein-coenzyme complexes. Furthermore the equilibrium ratio of alcohol to aldehyde was found to be 1350 to 1, when the concentrations of diphosphopyridine nucleotide and its dihydro derivative were equal. From this latter value the difference between the potential of the alcoholaldehyde couple and that of the diphosphopyridine couple can be calculated to be equal to .091 electron-volts at 20° C. and pH 7.9.

Negelein & Haas studied the analogous reaction between hexose monophosphate and triphosphopyridine nucleotide which produces phosphohexonic acid and dihydrotriphosphopyridine nucleotide. These spectrophotometric studies in the kinetics of enzyme reactions have shown that both the hydrogen donor and hydrogen acceptor form a complex with the protein, the hydrogen transfer very probably taking place within this complex. It is usually assumed that the hydrogen transfer takes place by a shifting of hydrogen atoms from one molecule to another (81). A more likely mechanism is that involving a dissociation of a hydrogen ion into the medium with the shifting of an electron from one molecule to the other, followed by the association of a hydrogen ion from the medium to its new position in the acceptor molecule. Unfortunately, since these kinetic experiments in-

volving single steps of the hydrogen transfer system have been made at only one temperature, calculations of the energies of activation cannot be made. Data of this kind should be available for a better insight into the process.

By a spectrophotometric method Haas (82) determined the velocity constants between oxidized "old" yellow enzyme and reduced di- and triphosphopyridine nucleotides. He showed further that, at 0° C. and in the presence of excess triphosphopyridine nucleotide and with an insufficient amount of hydrosulfite, a complex, which had a red color and of which he determined the absorption spectrum, could be obtained. From its spectral characteristics he presumed that the substance formed was similar to the flavin radical found by Kuhn & Wagner-Jauregg (83).

By measuring the rate of oxygen consumption and the rate at which cytochrome-c was reduced it was shown (84) that for yeast, depleted of substrate and at 0° C., the total respiration of yeast, under these conditions at least, proceeds through cytochrome-c.

Chase & Smith (85), using a photoelectric spectrophotometer designed specially for the measurement of the absorption of visual purple in the visible region (86), studied the rate of regeneration of this substance in solution after photochemical bleaching. These observers found that this reaction is first order with respect to the visual purple, except in a few instances for which the regeneration rate was abnormally high. Bleaching by blue or violet light gave greater subsequent regeneration than light lacking these components. This effect was ascribed to a photosensitive substance which absorbs at the shorter wave lengths. Successive regenerations were slower due to disappearance of the visual purple but the velocity constants remained unchanged.

Very rapid biochemical reactions have been measured by a photoelectric method involving the rapid flow of the mixed reagents. Hartridge & Roughton (87) measured the half time of combination of oxygen and hemoglobin and that for the decomposition of oxyhemoglobin. The latter reaction was found to be first order over a moderate range. The equilibrium constant calculated from the velocity constants for the forward and reverse directions was in agreement with that obtained by the static method. Their method was further developed by Roughton & Millikan (88) and by Millikan (89) and the latter used it to determine the kinetics of myoglobin (90). He found that, with the myoglobin concentration equivalent to that of blood diluted 150 times and the oxygen tension reduced to 60 mm. of mer-

cury, the process was 60 per cent completed in the first .0008 sec. The constant for this reaction was found to be about five times that for the analogous reaction for hemoglobin. The spectrophotometric flow method, as recently developed by Chance (91), is capable of measuring velocity constants for reactions which are half completed in a few ten-thousandths of a second while employing only one cubic centimeter of solution. This apparatus should prove very useful for studying the fast reactions involved in respiration.

LITERATURE CITED

1. SUHRMAN, R., AND KOLBATH, W., *Biochem. Z.*, **184**, 216 (1927)
2. ZSCHEILE, JR., F. P., *Cold Spring Harbor Symposia Quant. Biol.*, **3**, 108 (1935)
3. STOTZ, E., SIDWELL, JR., A. E., AND HOGNESS, T. R., *J. Biol. Chem.*, **124**, 11 (1938)
4. HOGNESS, T. R., ZSCHEILE, JR., F. P., AND SIDWELL, JR., A. E., *J. Phys. Chem.*, **41**, 379 (1937)
5. ASHLEY, S. E. Q., *Ind. Eng. Chem., Anal. Ed.*, **11**, 72 (1939)
6. VIERORDT, K., *Die Anwendung des Spectralapparates zur Photometrie der Absorptionspectren und zur Quantitativen Chemischen Analyze* (H. Laupp, Tuebingen, 1873)
7. HUFNER, G., *Arch. Anat. Physiol., Physiol. Abt.*, 39 (1900)
8. BRODE, W. R., *Chemical Spectroscopy* (Wiley and Sons, New York, 1939)
9. MILLER, E. S., *Quantitative Biological Spectroscopy* (Burgess Pub. Co., Minneapolis, 1939)
10. HAAS, E., *Biochem. Z.*, **282**, 224 (1935)
11. CUSTERS, J. F. H., *Z. tech. Physik*, **14**, 154 (1933)
12. HARRISON, G. R., *Proceedings of the Sixth Summer Conference on Spectroscopy and Its Application*, p. 91 (John Wiley and Sons, Inc., New York, 1939)
13. HARRISON, G. R., AND BENTLEY, E. P., *J. Optical Soc. Am.*, **30**, 290 (1940)
14. HAUSSER, K. W., *Z. tech. Physik*, **15**, 10 (1932)
15. HAUSSER, K., KUHN, R., AND COLLABORATORS, *Z. physik. Chem., Abt. B*, **29**, 363, 371, 378, 384, 391, 417 (1935)
16. MULLIKEN, R. S., *J. Chem. Physics*, **7**, 364, 570 (1939) ; McMURPHY, H. L., AND MULLIKEN, R. S., *Proc. Natl. Acad. Sci. U.S.*, **26**, 312 (1940)
17. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **287**, 291 (1936)
18. STERN, K. G., AND HOLIDAY, E. R., *Ber.*, **67**, 1442 (1934)
19. REICHSTEIN, T., *Helv. Chim. Acta*, **19**, 1107 (1936)
20. HOGNESS, T. R., SIDWELL, JR., A. E., AND ZSCHEILE, JR., F. P., *J. Biol. Chem.*, **120**, 239 (1937)
21. MURRAY, JR., W. M., AND ASHLEY, S. E. Q., *Ind. Eng. Chem., Anal. Ed.*, **10**, 1 (1938)
22. WICHMANN, H. J., *Ind. Eng. Chem., Anal. Ed.*, **11**, 66 (1939)
23. MILLER, E. S., MACKINNEY, G., AND ZSCHEILE, JR., F. P., *Plant Physiol.*, **10**, 375 (1935)
24. MILLER, E. S., *Plant Physiol.*, **12**, 667 (1937)

25. MILLER, E. S., *J. Am. Chem. Soc.*, **57**, 347 (1935)
26. MILLER, E. S., *Cereal Chem.*, **15**, 310 (1938)
27. MICHEL, H. O., AND HARRIS, J. S., *J. Lab. Clin. Med.*, **25**, 445 (1940)
28. CENTRAL SCIENTIFIC CO., *Circular No. 1137* (Chicago, 1940); COLEMAN ELECTRIC CO., *Circular on the Spectrophotometer* (Chicago)
29. EVELYN, K. A., AND MALLOY, H. T., *J. Biol. Chem.*, **126**, 655 (1938)
30. HOGNESS, T. R., ZSCHEILE, JR., F. P., SIDWELL, JR., A. E., AND BARRON, E. S. G., *J. Biol. Chem.*, **118**, 1 (1937)
31. SIDWELL, JR., A. E., MUNCH, R. H., BARRON, E. S. G., AND HOGNESS, T. R., *J. Biol. Chem.*, **123**, 335 (1938)
32. ALTSCHUL, A. M., SIDWELL, JR., A. E., AND HOGNESS, T. R., *J. Biol. Chem.*, **127**, 123 (1938)
33. ALTSCHUL, A. M., AND HOGNESS, T. R., *J. Biol. Chem.*, **129**, 315 (1939)
34. STOTZ, E., SIDWELL, JR., A. E., AND HOGNESS, T. R., *J. Biol. Chem.*, **124**, 11 (1938)
35. ALTSCHUL, A. M., AND HOGNESS, T. R., *J. Biol. Chem.*, **124**, 25 (1938)
36. KEILIN, D., AND HARTREE, E. F., *Proc. Roy. Soc. (London), B*, **127**, 167 (1939)
37. POTTER, V. R., *J. Biol. Chem.*, **137**, 13 (1941)
38. WARBURG, O., *Ergeb. Enzymforsch.*, **7**, 210 (1938)
39. THEORELL, H., *Biochem. Z.*, **285**, 207 (1936)
40. JUNOWICZ-KOCHOLATY, R., AND HOGNESS, T. R., *J. Biol. Chem.*, **129**, 569 (1939)
41. POTTER, V. R., *Medicine*, **19**, 441 (1940)
42. WARBURG, O., CHRISTIAN, W., AND GRIESE, A., *Biochem. Z.*, **282**, 157 (1935)
43. EULER, H. v., ADLER, E., AND HELLSTRÖM, H., *Z. physiol. Chem.*, **241**, 239 (1936)
44. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **287**, 291 (1936)
45. GREEN, D. E., NEEDHAM, D. M., AND DEWAN, J. G., *Biochem. J.*, **31**, 2327 (1937)
46. EULER, H. v., ADLER, E., GÜNTHER, G., AND HELLSTRÖM, H., *Z. physiol. Chem.*, **245**, 217 (1937)
47. ADLER, E., EULER, H. v., AND HUGHES, W., *Z. physiol. Chem.*, **252**, 1 (1938)
48. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **303**, 40 (1939)
49. ADLER, E., AND GÜNTHER, G., *Z. physiol. Chem.*, **253**, 143 (1938)
50. EULER, H. v., ADLER, E., GÜNTHER, G., AND DAS, N. B., *Z. physiol. Chem.*, **254**, 61 (1938)
51. EULER, H. v., ADLER, E., AND GÜNTHER, G., *Z. physiol. Chem.*, **247**, 65 (1937)
52. HAAS, E., HORECKER, B. L., AND HOGNESS, T. R., *J. Biol. Chem.*, **136**, 747 (1940)
53. NEGELEIN, E., AND HAAS, E., *Biochem. Z.*, **282**, 206 (1935)
54. ADLER, E., EULER, H. v., AND GÜNTHER, G., *Skand. Arch. Physiol.*, **79**, 240 (1938)
55. ADLER, E., EULER, H. v., GÜNTHER, G., AND PLASS, M., *Biochem. J.*, **33**, 1028 (1939)
56. ADLER, E., EULER, H. v., AND GÜNTHER, G., *Nature*, **143**, 641 (1939)

57. OHLMEYER, P., *Biochem. Z., 297,* 66 (1938)
58. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z., 298,* 368 (1938)
59. EULER, H. V., AND GÜNTHER, G., *Naturwissenschaften, 26,* 676 (1938)
60. EULER, H. V., AND GÜNTHER, G., *Z. physiol. Chem., 256,* 229 (1938)
61. EULER, H. V., AND HAASE, K., *Naturwissenschaften, 26,* 187 (1938)
62. EULER, H. V., AND HELLSTRÖM, H., *Z. physiol. Chem., 252,* 31 (1938)
63. ADLER, E., EULER, H. V., GÜNTHER, G., AND PLASS, M., *Skand. Arch. Physiol., 82,* 61 (1939)
64. LOCKHART, E. E., AND POTTER, V. R., *J. Biol. Chem., 137,* 1 (1941)
65. URBAN, F., AND PEUGNET, H. B., *Proc. Roy. Soc. (London), B,* 125, 93 (1938)
66. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z., 266,* 377 (1933)
67. HAAS, E., *Biochem. Z., 298,* 378 (1938)
68. NEGELEIN, E., AND BRÖMEL, H., *Biochem. Z., 300,* 225 (1939)
69. BALL, E. G., *J. Biol. Chem., 128,* 51 (1939)
70. STRAUB, F. B., *Biochem. J., 33,* 787 (1939)
71. CORRAN, H. S., AND GREEN, D. E., *Nature, 142,* 149 (1938)
72. KEILIN, D., AND HARTREE, E. F., *Proc. Roy. Soc. (London), B,* 122, 298 (1937)
73. THEORELL, H., *Biochem. Z., 285,* 207 (1936)
74. KEILIN, D., *Ergeb. Enzymforsch., 2,* 239 (1933)
75. THEORELL, H., *Biochem. Z., 288,* 317 (1936)
76. EULER, H. V., AND HELLSTRÖM, H., *Z. physiol. Chem., 255,* 159 (1938)
77. ALTSCHUL, A. M., ABRAMS, R., AND HOGNESS, T. R., *J. Biol. Chem., 136,* 777 (1940)
78. BALL, E. G., *Biochem. Z., 295,* 262 (1938)
79. MILLIKAN, G. A., *Proc. Roy. Soc. (London), B,* 123, 218 (1937)
80. NEGELEIN, E., AND WULFF, H.-J., *Biochem. Z., 293,* 351 (1937)
81. OPPENHEIMER, C., AND STERN, K. G., *Biological Oxidation* (Uitgeverij Dr. W. Junk, The Hague, 1939)
82. HAAS, E., *Biochem. Z., 290,* 291 (1937)
83. KUHN, R., AND WAGNER-JAUREGG, T., *Ber., 67,* 361 (1934)
84. HAAS, E., *Naturwissenschaften, 22,* 207 (1934)
85. CHASE, A. M., AND SMITH, E. L., *J. Gen. Physiol., 23,* 21 (1939)
86. SCHLAER, S., *J. Optical Soc. Am., 28,* 18 (1938)
87. HARTRIDGE, H., AND ROUGHTON, F. J. W., *Proc. Roy. Soc. (London), A,* 104, 376 (1923)
88. ROUGHTON, F. J. W., AND MILLIKAN, G. A., *Proc. Roy. Soc. (London), A,* 155, 258 (1936)
89. MILLIKAN, G. A., *Proc. Roy. Soc. (London), A,* 155, 277 (1936)
90. MILLIKAN, G. A., *Proc. Roy. Soc. (London), B,* 120, 366 (1936)
91. CHANCE, B., *J. Franklin Inst., 229,* 455, 613, 741 (1940)

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REVIEW OF BIOLUMINESCENCE

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Luminescence of living things is a chemiluminescence in which some of the energy from catalytic oxidation of a definite organic compound is emitted as light. Luciferin is oxidized in the presence of the enzyme, luciferase. The author (4) has recently published a book, *Living Light,* dealing with all aspects of bioluminescence and some inorganic luminescences, largely from the historical standpoint. Consequently, this review will include only the papers of special physiological and biochemical interest from 1935 to 1940, so far as the literature was available. During this period, the attention of investigators has been largely directed to the chemical nature of the luminous substances, and to the relation between luminescence and cell respiration, in an attempt to correlate the light-giving luciferin and luciferase system with other well-known respiratory systems.

BIOLOGICAL STUDIES

During the last five years one new order of luminous animals has been added to the forty orders already known to produce light, by Kato's discovery of a luminous nemertean, *Emplectonema kandai.* Its surface luminesces when stimulated, although no luminous slime is excreted that sticks to the fingers. The dried animal luminesces when again moistened but the presence of luciferin and luciferase cannot be demonstrated (Kanda). A new species of luminous bacteria, *Achromobacter harveyi,* has been described by Johnson & Shunk; and new studies have been made of light production and luminous cells of the marine worm, *Chaetopterus* (Fujiwara), of earthworms (Backovsky, Komarek & Wenig; Komarek & Wenig), of collembolid insects (Heidt), and fly larvae (Fulton), as well as new detailed histological studies on the ostracod crustacean, *Cypridina* (Takagi), Japanese fireflies (Okada), and *Salpa* (Stier). The suggestion by Parker that the lizard, *Proctoporus shrevei,* contains luminous organs is probably not correct. Bothe has studied extensively the heredity of luminescence in the fungus, *Mycena,* in which cultures from single spores show all degrees of luminosity. He finds that luminescence is affected by three series of hereditary factors, some of which are inhibitors. Macrae

noted that pairings of the nonluminous and luminous strains of the fungus, *Panus stipticus,* gave luminescent mycelia, i.e., luminescence was dominant.

PHYSIOLOGICAL STUDIES

Among fireflies, the new work has dealt largely with synchronous flashing and the mechanism of flashing. Synchronous flashing is particularly characteristic of tropical fireflies, where all the individuals on one tree may flash at once. Alexander noted a leader but others have not been able to discern a definite pacemaker. The meaning of synchronism in tropical forms is obscure since Smith reported that all the flashing individuals on trees are males and may keep up their synchronism for days, stopping only in moonlight or daylight. The females lie hidden in the forest. North American fireflies have been extensively studied by Buck (1, 2, 4, 5) who occasionally noted some synchronous flashing, due to the fact that the light is a mating signal. When a male flashes, the female responds with a flash exactly two seconds after the male flashes. Several males may be attracted to the female and this little group then acts as a unit in setting off another female. Quite a number may become synchronous by this process. Buck (3) has studied the effect of light on flashing, diurnal rhythm, and periodicity of flashing, as well as color vision in the firefly. He found that males will respond to a flash of varying intensity and duration and of any color from green to deep red (not blue green or blue) provided the time interval is two seconds after a previous flash.

Ruedemann has noted a simultaneous flashing in American fireflies due to a pressure wave from exploding firecrackers.

The mechanism of flashing in the firefly has always centered around two theories: (*a*) that nerves which go to the organ stimulate photogenic cells directly, as a nerve stimulates a muscle; (*b*) that nerves control a valve mechanism at the ends of the tracheae, thus suddenly admitting more air to the organ, with a resultant flash, since the luminescence is dependent on oxygen. Maluf (1, 2) has presented a third theory of flashing. He believes that nerves and fluid-filled tracheoles both enter photogenic cells. Nerve stimuli cause production of metabolites (lactic acid) whose osmotic pressure draws fluid from the tracheoles, thus allowing oxygen to enter the cell and cause luminescence. Oxidation of the lactic acid lowers the osmotic pressure and fluid again fills the tracheoles with extinction of light. Maluf has found that injecting hypertonic solutions into the abdomen results

in a continuous glow, but isotonic or hypotonic solutions do not. The continuous glow at low oxygen tensions has been attributed to accumulation of metabolites, and that at high oxygen tension to continual diffusion of oxygen into the organ.

Emerson studied the effect of ether on the firefly, *Photuris pennsylvanica.* He found that ether does not affect the luminescence of animals whose glow was made continuous by previous injections of adrenalin, unless lethal concentrations are used. In animals with flashing mechanism intact, ether does not affect the flash until motility ceases, when a constant luminescence appears. Irreversible quenching of this constant light only occurs with lethal concentrations. Therefore ether affects first the motility of the animal, then the flashing mechanism, and finally the chemiluminescent systems themselves. Wood observed that spider poison caused scintillation of the light organ of the firefly.

One of the most interesting recent developments in firefly physiology is the recording of an "electroluminogram" the potential change accompanying a flash in *Luciola cruciata.* Hasama obtained string galvanometer records showing a monophasic action potential during each flash which was not due to breathing or any muscle movement. It was larger in oxygen and disappeared in ether narcosis when the luminescence ceased. It would be interesting to record, simultaneously with this electrical change, the course of the luminescence intensity–time curve, using a photocell for detection, as in the experiments of Brown & King (1931)[1] and Snell (1931–32).

RELATION TO OXYGEN

Although the great majority of luminous animals require molecular oxygen for luminescence, the author noted in 1926 that radiolaria, ctenophores and the medusa, *Pelagia,* do not require it. This remarkable finding was confirmed by Harvey & Korr for the ctenophore, *Mnemiopsis leidyi,* under the most carefully controlled conditions for removing the last traces of oxygen.

Extracts or fragments of the photogenic cells of this animal lose their ability to luminesce in a strong light, whether oxygen is present or absent. The "light adapted" fragments recover ability to luminesce in the dark only if oxygen is present, not in its absence. This suggests

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[1] Authors' names followed by the year in parentheses refer to work which was done previous to 1935, and for which references are not included in the bibliography.

that the photogenic material of this form contains bound oxygen which is used up in luminescence or after exposure to light. Consequently no re-formation of photogenic material can occur in the dark if oxygen is absent.

Van Schouwenburg has confirmed, on *Photobacterium phosphoreum*, the earlier work of Shoup (1929) and Shapiro (1934), that both oxygen consumption and luminescence are independent of oxygen tension over a wide range, the luminescence being affected only at very low tensions.

CHEMISTRY OF THE LUMINOUS SUBSTANCES

The isolation and purification of the oxidizable compound, luciferin, and the oxidizing enzyme, luciferase, can be successfully carried out only in an animal such as the ostracod crustacean, *Cypridina*, in which the luminous substances are formed in a rather large gland whose secretion is extruded into the sea water. By drying the animals quickly, the gland can be preserved indefinitely; a bright luminescence occurs on moistening. Anderson (1) has contributed greatly to knowledge of bioluminescence by highly purifying the luciferin. At one stage in the process the luciferin is quite stable and not easily autooxidizable. The method involves the extraction of the dry Cypridinas with methyl alcohol. Ten per cent of butyl alcohol is then added and the methyl alcohol removed *in vacuo*. The supernatant butyl alcohol extract is chilled and benzoylated with benzoyl chloride. After fifteen minutes this solution is diluted with ten volumes of water and the new inactive benzoylluciferin derivatives extracted with pure ether. After removing the ether *in vacuo* the residual liquor is hydrolyzed with hydrochloric acid in absence of oxygen. The free active luciferin is left in the acid solution and can be extracted with butyl alcohol. By repeating the benzoylation and hydrolysis, a product purified 2,000-fold, as compared with dry Cypridinas, can be obtained.

To purify luciferase it is usually sufficient to dialyze a cold, well-stirred water extract of rapidly dried, powdered Cypridinas against cold running water for twenty hours. As shown by Giese & Chase, dialysis removes pigment, and a precipitate forms which can be filtered off. A few drops of toluene added to this solution will preserve it for months with little loss in activity if kept in a refrigerator.

Anderson had previously (1934) developed methods for quantitative determination of both luciferin and luciferase: for luciferin, by measuring the total light emitted under constant conditions; and for

luciferase, by measuring the rate of light emission. During purification procedures the yield could therefore be followed quantitatively.

It early became apparent that the total light emitted from a given quantity of luciferin and luciferase varied with the temperature (decreases 2 to 3.5 per cent per degree C. increase in temperature), the pH (decreases with greater alkalinity, above pH 6.0), and the salt content of the medium [Anderson (1)]. Anderson (3) studied salt effects in detail and found that the total light emitted from a certain amount of luciferin increased regularly with increasing sodium chloride concentration. Taking the total light emitted with no salt as 1, the following figures give the light for various salts in 0.0095 M concentration: sodium chloride, 2.3; potassium chloride, 2.2; sodium bromide, 1.9; potassium bromide, 2.0; potassium fluoride, 1.4; potassium nitrate, 1.3; potassium oxalate, 1.2; potassium sulfate, 1.1; potassium thiocyanate, 0.12; potassium iodide, 0.08. The effect is chiefly connected with the anion and the quenching effects of iodide and thiocyanate are largely eliminated by the presence of sodium chloride, the most effective salt studied.

When luciferin is oxidized, some product is formed, called oxyluciferin by the author (1918), which can be reduced to luciferin again. Anderson (2) has shown that the oxidation with production of light in the presence of luciferase gives an oxidation product which cannot be reduced, whereas the oxidation without luminescence by oxidants like potassium ferricyanide is reversible. This product is called oxidized luciferin by Anderson (2). The change with ferricyanide occurs in two steps, one of which is the reversible oxidation previously referred to; the second is irreversible and probably also an oxidation, although this has not been definitely demonstrated. The well-known spontaneous oxidation of luciferin without emission of light in crude solutions (without luciferase) is probably catalyzed by traces of heavy metals in the solution and proceeds much more slowly when the luciferin has been purified. Both the nonluminescent oxidation and the luminescent oxidation undoubtedly take place simultaneously when luciferin is mixed with luciferase.

If much reversibly oxidized luciferin is present together with luciferin, added luciferase will irreversibly oxidize the luciferin quickly with a bright luminescence, and then a weak long-lasting luminescence may appear. The latter is presumably due to reduction of the reversibly oxidized luciferin, as the equilibrium is upset by irreversible luciferase oxidation, which continues slowly with the production of a

weak light. The redox potential of the first step in oxidation has been placed by Anderson (2) near the hydroquinone $\rightleftharpoons$ quinone system but 0.01 to 0.005 volts more negative. Korr (3) has also placed it near quinone. If we assume a two electron change, the redox potential would be $E'_0 = +0.26$ volts at pH 7.0, about halfway between quinone and orthochlorphenolindophenol.

The availability of pure luciferin has made possible a more exact determination of the properties of this substance. The author (1925) had observed that in crude extracts of whole Cypridinas the luciferin underwent rapid spontaneous oxidation in strong light (without luminescence).[2] Chase has recently found that the effect of light in accelerating oxidation of luciferin is dependent on the presence of a naturally occurring fluorescent pigment, possibly a flavin. The light of a luminescent mixture of purified luciferin and luciferase was not quenched by strong illumination unless boiled extract of Cypridinas or pure riboflavin had been added. Eosin and fluorescein also sensitized quenching of the luminescence of purified luciferin.

Chase & Giese have also studied the effect of ultraviolet light on purified luciferin which was free of substances which might absorb ultraviolet and consequently protect the luciferin. In ultraviolet of wave length 230 to 300 mμ. destruction occurs whether oxygen is present or not. The effect of short-wave ultraviolet irradiation therefore appears to be different from that of visible light since the author has shown that visible light affects luciferin only in the presence of oxygen.

The absorption of visible light at several wave lengths by highly purified solutions of luciferin has been studied by Chase, who found that during the first stages of spontaneous nonluminescent oxidation an absorption band with maximum at about 470 mμ. appeared. During further oxidation the solution became almost colorless. The rate of these color changes paralleled the oxidation of luciferin in water at different hydrogen ion concentrations, as well as in butyl alcohol as a solvent.

Measurements of the absorption spectrum made by Chase (unpublished) with the Hardy and with the Harrison recording spectrophotometers show: (a) that freshly dissolved luciferin has an absorption

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[2] The absence of luminescence can be established by illumination of an eosin-sensitized luminescent *Cypridina* extract with intense yellow-green light while observing the tube through a blue filter. No increased luminescence can be detected.

maximum at 430 mμ., or slightly lower, and is yellow in color; (b) that during oxidation this absorption maximum is rapidly replaced by one at 470 mμ., which subsequently decreases to give a colorless solution; (c) that in the presence of luciferase these same color changes also occur, but at least one hundred times faster; (d) that the amount of luciferin in a solution (as measured by total luminescence) is directly proportional to the labile color of the solution and not to the total color, and (e) that the ultraviolet absorption decreases in the region 250 to 300 mμ. during the first stages of nonluminescent oxidation and subsequently increases in the region 320 to 420 mμ.

The ultraviolet absorption spectrum of freshly dissolved luciferin shows inflexions suggesting maxima at 310 to 320 mμ., 280 mμ., and 260 mμ., with practically complete absorption at 240 mμ. and below (Chase & Giese). If all the absorption is due to luciferin, a benzene or naphthalene structure may be indicated.

Luciferin is thus certainly to be identified with the yellow granules of the gland cells of *Cypridina*. A marked yellow color is also present in the luminous glands of many luminous animals—in the worm, *Tomopteris*, in the medusae, *Aequorea* and *Mitrocoma*, and in copepods. The luminous organ of the firefly, of *Pyrophorus* and *Pyrosoma*, the slime of myriapods and of the fish, *Malacocephalus*, are yellowish. All attempts to demonstrate carotenoid pigments have failed but it is very probable that some flavin is present and possibly concerned in light production, although it is unlikely that luciferin from *Cypridina* is itself a flavin.

The author (1926) has repeatedly observed that the luminous glands of many forms are markedly fluorescent in ultraviolet light. This is particularly true of fireflies, the elaterid beetle, *Pyrophorus*, ophiurans, luminous earthworms, the marine worm, *Acholoë*, copepods and ctenophores, but not of *Cypridina hilgendorfii*, whether the luminous substances are irradiated in acid, neutral, or alkaline solutions.

Recently, Komarek & Wenig, and Backovsky, Komarek & Wenig have studied the yellow-green luminescence of yellow granules in the lymphocytes of the earthworm, *Eisenia submontana*. The worm is not luminous on gentle handling but when strongly irritated (on lethal stimulation) the lymphocytes are extruded through the dorsal pores with luminescence. These authors have reported that the luciferin-luciferase reaction can be obtained and that the granules are not luminous bacteria, but slowly dissolve as luminescence appears. Oxygen is

necessary and potassium cyanide does not affect the light. The oxidized luciferin cannot be reduced. In a nonluminous earthworm, *Eisenia foetida,* similar yellow granules in the lymphocytes are nonluminous although they fluoresce yellow-green, becoming blue in ultraviolet light. This is also true of the yellow granules in the luminous *E. submontana.* They fluoresce yellow-green in ultraviolet light, become blue fluorescent after they have lost their bioluminescence. These authors believe that the fluorescent yellow granules contain lactoflavin (which they verified by chemical analysis and by recording the fluorescent spectrum) which changes to lumiflavin both as a result of luminescence and after exposure to ultraviolet light. They regard the bioluminescence of this earthworm as really a fluorescence due to special chemical processes in the yellow granules, which give rise to short-wave rays. In the first paper these are called mitogenetic rays. Brooks has found a flavoprotein in the luminous segments of the glowworm.

The chemical nature of luciferin is as yet unknown but it is probably not a proteose or a phospholipin, as had been previously supposed. Anderson (1) and Korr (3) believe that it may be one of the polyhydroxybenzenes, many of which are oxidized in two steps, and have redox potentials in the same region as that of the luciferin system.

In general cyanide has no or little (in luminous bacteria) effect in suppressing bioluminescences. The author (1917) observed no inhibition of the luminescence of extracts of whole Cypridinas, even by 0.1 M potassium cyanide when the medium was sufficiently buffered to prevent undue alkalinity. However, Giese & Chase found that luciferin purified by the method of Anderson is affected by low cyanide concentration in such a way that no light appears upon addition of luciferase. The luciferin-cyanide combination appeared irreversible. Purified solutions of luciferase were not affected by cyanide. At concentrations of cyanide below those which completely prevent luminescence, light emission varied inversely with cyanide concentration, suggesting a combination of cyanide with luciferin. Although impurities as well as luciferin may combine with cyanide, and the data do not indicate a simple chemical combination, calculations of the combining weight indicate that the luciferin molecule may be relatively small, of molecular weight less than 1,000.

The experiments suggest that *Cypridina* luciferin in the crude extracts is combined with another compound, possibly a protein, through the linkage (perhaps an aldehyde or ketone) which is attacked by cyanide. Hence in crude extracts cyanide does not suppress lumines-

cence. The purification of luciferin may cause a splitting of luciferin from its protein (?) combination. Another fact is in favor of this idea. Harvey (1917–19) found that ether and benzene will not extract luciferin from dried Cypridinas but Kanda (1929) observed that luciferin becomes soluble in ether and benzene after previous treatment with alcohols.

KINETICS OF LUMINESCENCE

The kinetics of the decay of luminescence when *Cypridina* luciferin and luciferase are mixed are well known since the work of Amberson (1922). Kinetics of the rate of appearance of light have recently been worked out by Chance, Harvey, Johnson & Millikan, using an accelerated flow method, a microphotoelectric form of the Hartridge-Roughton flow technique. They found that the time to reach half the maximum intensity is 0.006 second and full intensity 0.03 second. The half time of decay in these ultrashort flashes may be only 0.12 second. The decay curve is logarithmic in form.

The above statement applies to conditions where oxygen is already present. If luciferin and luciferase are previously mixed in absence of oxygen and then this anaerobic solution mixed with areated water, the time required to attain to half the maximum intensity is 0.002 second, and full intensity 0.008 second, three times as fast. The experiment clearly shows that luciferin and luciferase combine slowly, whereas the combination with oxygen is rapid.

Apparently four reactions in series are involved. The steps may be represented as follows, a combination of the schemes of Harvey (1, 2) and of Johnson, van Schouwenburg & van der Burg.

(1) LH_2 (luciferin) $+ A$ (luciferase) $\rightarrow A \cdot LH_2$
(2) $A \cdot LH_2 + 1/2\, O_2 \rightarrow A \cdot LH_2 \cdot O$
(3) $A \cdot LH_2 \cdot O \rightarrow A'$ (excited luciferase) $+ L$ (oxidized luciferin) $+ H_2O$
(4) $A' \rightarrow A + h\nu$ (a quantum of light)

Mathematical analysis indicates that two consecutive monomolecular reactions with the velocity constants, k_3 and k_4, will explain the course of the intensity of the experimental flash with time.

Chance, Harvey, Johnson & Millikan also have studied the flash of light or excess luminescence when luminous bacteria in absence of oxygen are suddenly mixed with aerated sea water. They have found that with adequate oxygen the luminescence rises to a high intensity and then falls to the plateau level of uniform intensity, characteristic of these bacteria. The time necessary to reach half-maximum inten-

sity is 0.08 second and peak intensity, 0.34 second, very much slower than the corresponding process for *Cypridina* luminescence. Because oxygen tensions varying between 380 mm. and 10 mm. Hg do not alter the time relation, it would seem as if the slow rate of appearance of luminescence in bacteria is a characteristic of the oxidation in these forms and not due to time of diffusion of oxygen through the cell surfaces. These results can also be analyzed in terms of two consecutive monomolecular reactions, equations 3 and 4, with velocity constants k_3 and k_4.

Johnson & Lynn (unpublished) have found in the fungus, *Panus stipticus,* a similar flash when air is admitted after anaerobiosis, the duration of which is some ten times longer than that of bacteria.

Recently, Schoepfle (1), using a new method of recording (oscillator, balanced photocell bridge circuit, amplifier, cathode ray oscillograph), has studied the flash of luminous bacteria at different temperatures. The characteristics (form and area) of the flash curves depend on the temperature at which the light is produced, not on the temperature at which the bacteria are held before mixing with oxygen. At all temperatures, constants for equations 3 and 4 fit the curves; k_3 varies with temperature and gives a μ. value of 8,000 Cal. when analyzed by the Arrhenius equation; k_4 is independent of temperature. The total light in the flash decreases as the temperature is raised (12 to 27° C.), an effect which may be due to (*a*) a shift of the equilibruim, $LH_2 + A \rightleftharpoons A \cdot LH_2$, with temperature; (*b*) a two-way breakdown of $A \cdot LH_2 \cdot O$ to yield both excited and unexcited molecules, the relative proportions of which vary with temperature; or (*c*) possible ionic or molecular quenching which varies with temperature. It is not possible to distinguish between these effects from the change of intenstiy with time alone.

Schoepfle (2) has also found no change in k_3 and k_4 in hypotonic solutions or in veronal or dinitrophenol, but k_3 was lowered in hypertonic solution, due possibly to increased viscosity within the bacterial cell.

STUDIES ON LUMINOUS BACTERIA

Luminous bacteria offer convenient material for luminescence investigations since they can be easily grown the year round on ordinary culture media. The disadvantage in using them lies in the fact that the luciferin-luciferase reaction cannot be demonstrated, despite many

attempts to do so. Korr (1) has tested several new methods of extraction, including supersonic disintegration in absence of oxygen, without success. No luminous substances could be extracted which would luminesce in a test tube. Ballentine & Harvey (unpublished) tried rapid freezing with evaporation of ice *in vacuo* with negative results. All procedures which destroy the bacterial cell also destroy its ability to luminesce. However, all investigators assume that luciferin and luciferase are present. Research has largely been directed to a simultaneous study of respiration and luminescence, with particular reference to the action of foodstuffs, respiration accelerators or poisons, and certain unfavorable conditions.

Salts.—Johnson & Harvey (1) investigated the behavior of marine luminous bacteria in diluted and concentrated sea water. These bacteria do not swell in greatly diluted sea water but are apparently surrounded by a rigid membrane which cracks [Hill (1929)], letting out some of the contents of the cell. At the same time the luminescence disappears. In moderately diluted sea water, luminescence falls off practically in proportion to dilution but respiration may continue at the normal high rate in 50 per cent sea water. In sea water which has been concentrated to different degrees, respiration falls off with increasing concentration, but in sea water which is twice the ordinary concentration the luminescence may be little affected.

Many experimenters have studied the growth of luminous bacteria with different salts in the medium. The growth is often remarkably independent of the kind of salt used, provided the osmotic pressure is maintained the same. The detailed results of these researchers are so conflicting, however, that it is not possible to draw any very definite conclusions. Mostly the investigations have been carried out by growing the organisms in culture media whose sodium chloride has been replaced by other salts.

The most sensitive method of studying salt effects is to measure respiration and luminescence of the bacteria. Johnson & Harvey (2) have found the general respiration of well-washed suspensions of *Achromobacter fischeri* to be practically the same in isotonic sodium phosphate, sea water, and pure $0.5 M$ sodium chloride, but diminished in isotonic potassium chloride, sucrose, and magnesium chloride, and practically abolished in calcium chloride. Luminescence is generally more sensitive than respiration. It is almost quenched in calcium or magnesium chloride, greatly affected in potassium chloride, and somewhat affected in sucrose.

The results of Claren are not entirely in agreement. He used well-washed suspensions and studied respiration (but not luminescence) of *Micrococcus cyanophos* in different concentrations of salt. Solutions of sodium chloride, 1.75 per cent, containing a little glucose proved to be the optimum concentration. In glucose solutions of the same osmotic pressure but without salt, respiration was cut about in half. The sodium ion could be replaced by lithium, potassium, or ammonium ions, and the chloride ion by bromide or iodide ions without great changes in respiratory rate, but the ions did have an effect that ran parallel to ionic size. Replacing one quarter of the sodium chloride with a phosphate buffer mixture (pH 6.98) increased the oxygen consumption, and adding a little magnesium chloride to this had a still further beneficial effect which was not apparent when magnesium was added to sodium chloride alone. The respiration in different salts depends on the substrate and is decidedly complicated. Van Schouwenburg found that $0.1 \, M$ phosphate buffer, at pH 7.6, gave optimum oxygen consumption by *Photobacterium phosphoreum*. Johnson, van Schouwenburg & van der Burg used equal parts of 3 per cent sodium chloride and $0.25 \, M$ phosphate buffer at pH 7.3, called "PN," as a favorable suspending medium for *Photobacterium fischeri*. It allowed a long-lasting luminescence with rapid respiration.

Takase noted a difference in spectral distribution of the light when bacteria from the light organ of a deep-sea fish, *Coelorhynchus kishinouyei,* are grown on $0.8 \, M$ sugar as compared with $0.5 \, M$ sodium chloride.

Bukatsch studied the effect of salts on luminescence in an amino acid medium and found that sodium and potassium are essential. Zinc and aluminum are inhibitory but calcium, strontium, and barium can counteract their effect.

Temperature.—Temperature-luminescence curves are well known. Recently Akabane, using luminous bacteria isolated from deep-sea fish of the family Macrouridae has recorded another such curve in which the temperature of maximum light is 27° C., falling off at higher and lower temperatures. Schoepfle's (1) studies on *Achromobacter fischeri* also gave a maximum of 27° C. Eymers & van Schouwenburg (2) found that the quantum efficiency of luminescence of *Photobacterium phosphoreum* varied with temperature as described on page 549.

Hydrogen ion concentration.—Luminous bacteria respire and luminesce well between pH values of 5.9 and 8.3, a normal range (Claren) and Eymers & van Schouwenburg (1) were able to photo-

graph the luminescent spectra in phosphate buffers between pH 5.3 and 8. Van Schouwenburg found little change in oxygen consumption between pH 5.7 and 8.

Ultraviolet light.—The effects of ultraviolet light have been studied by Giese, who was unable to find any marked stimulation of either respiration or luminescence, as has been claimed by some workers. After a certain time, irradiated luminous bacteria show a decline in respiration and luminescence which is proportional to dosage of radiation. The reproductive mechanism is more readily affected than the general respiration, and oxidation chains resulting in luminescence are more readily affected than those involved in general respiration.

Respiratory accelerators.—If bacteria are supplied with ample food, there are few substances which will increase the respiratory rate and none that will increase the luminescence; with little food material, many substances can increase the low respiration and luminescence. In order to study properly the effect of food, the washed-cell technique must be used. Luminous bacteria are thoroughly washed with sea water until only a low "endogenous" respiration and luminescence remain. Then the material to be studied is added. The method has been extensively used by Johnson (1 to 5), who has investigated a long list of sugars and related compounds. If the sugar can be utilized, the respiration and luminescence rise immediately when the sugar is added to the washed bacteria. Only three- and six-carbon sugars and their polyhydric alcohols have been found to be oxidized by *Vibrio phosphorescens* and *Achromobacter fischeri*. It is well known that dyes like methylene blue or substances like dinitrophenol can increase the respiration of luminous bacteria. Dinitrophenol does not increase the luminescence. Hsu noted a slight increase in luminescence of suspensions of *Coccobacillus coelorhynchus* when negatively ionized air was drawn through the suspensions but not with positively ionized air. These experiments should be repeated. No effects were produced on growth by either positively or negatively ionized air.

Occasionally a strain of bacteria will become nonluminous. The question may be raised as to what is lacking in dim or dark strains. Perhaps it is some constituent necessary for the formation of luciferin or luciferase. Doudoroff has tested this viewpoint by studying the ability of lactoflavin (vitamin B_2), the prosthetic group of the yellow respiratory enzyme, to restore luminescence in dim strains. He found that some of the semidark dissociates of bright luminous bacteria did have their luminescence increased by small amounts of lacto-

flavin whereas others did not. The results were not as regular as one might wish.

Studies of isolated enzyme systems from luminous bacteria are urgently needed. Nakamura claims that *Micrococcus phosphoreus* contains little or no catalase and that luciferin destroys the hydrogen peroxide which accumulates. His conclusions sound unlikely, especially since van Schouwenburg (2) has found catalase in expected concentrations in seven different species of luminous bacteria.

LUMINOUS BACTERIA. RESPIRATORY POISONS

By studying the poisons which inhibit certain known steps in the series of respiratory oxidations, it is often possible to draw conclusions concerning the relation of luminescence to these steps. It is well known that some substances diminish luminescence without affecting respiration (total oxygen consumption), while others greatly affect respiration without much effect on luminescence.

Harvey & Taylor (1934) had found that heavy water (D_2O) reduced both the luminescence and respiration of salt and fresh water bacteria. According to Zirpolo heavy water affects the subsequent growth of *Bacillus pierantoni,* so that the bacteria develop more slowly and luminesce less strongly if first kept in 99.6 per cent heavy water. After two days' treatment the bacteria will grow but not luminesce in normal culture media.

Rerabek & Hykesova find that radium emanation (1.5 to 5 millicuries) diminishes the luminescence of cultures of *Vibrio phosphorescens.*

Of special interest are the effects on luminescence produced by (*a*) lipid-soluble narcotics, (*b*) cyanide, (*c*) carbon monoxide, (*d*) azide, (*e*) pyrophosphate, (*f*) arsenite, and (*g*) iodoacetate.

Lipid-soluble narcotics.—The lipid-soluble narcotics possess the ability to greatly affect the luminescence of bacteria, without lowering the oxygen consumption. This evidence as well as other facts indicate that the oxygen consumed for luminescence is a negligible per cent of the total respiration. Taylor has made a special study of ethyl urethane concentration and the per cent inhibition of both respiration and luminescence, in an unsuccessful attempt to apply the adsorption laws of Freundlich and Langmuir to the surface of the oxidative catalysts.

Fisher & Stern (unpublished) have applied the mass action law

to the urethane data of Taylor and of van Schouwenburg, making the assumption that the urethane combined with enzyme is represented by the per cent inhibition of the respiration, I, while enzyme concentration is measured by per cent uninhibited respiration, U. Then

$$\frac{U}{I} \, [\text{Urethane}]^a = K$$

and log U/I plotted again log urethane concentration should give a straight line whose slope is a, the number of molecules which combine with the enzyme. Actually two interacting straight lines were found, interpreted to mean that the respiration is made up of two quantitatively distinguishable fractions. Similar treatment of cyanide and azide data also yield straight lines and varying values of a. In general the findings support the assumption of a combintaion of inhibitor with one or more of the oxidative catalysts within the cell.

Johnson (5) studied the inhibition of methylene blue reduction by luminous bacteria in the presence of ethyl ($0.2 \, M$) and n-butyl urethane ($0.01 \, M$) and Johnson & Chambers the methylene blue reduction, oxygen consumption and luminescence of *Achromobacter fischeri* by sodium diethylbarbital (veronal). At various concentrations the per cent inhibition of respiration parallels the per cent inhibition of methylene blue reduction but the per cent inhibition of luminescence is very much greater. The veronal must attack luciferase specifically; in higher concentration it obviously affects the other dehydrogenases. Van Schouwenburg's studies on urethane and on combinations of urethane and cyanide, which mutually reinforce each other's action, indicate action of the urethane on the light emitting reaction. Further evidence for this conclusion is found in the effect of urethane in greatly diminishing the "flash" or excess luminescence which occurs when oxygen is admitted to bacterial suspensions kept under anaerobic conditions for some time (Johnson, van Schouwenburg & van der Burg). By this type of experiment, it is possible to study effects on the light-emitting reaction itself (i.e., on the oxidation of a store of excess luciferin), as contrasted with effects on secondary reactions which may be concerned in the formation of luciferin from some precursor (proluciferin), or the reduction of an oxidized form of the compound by hydrogen donors such as glucose, succinate, etc.

Cyanide.—The action of cyanide has been extensively studied. Luminous bacteria have their oxygen consumption greatly reduced while the light intensity is affected to a far less degree. There is still

a small oxygen consumption when luminescence has been reduced to zero. By plotting per cent reduction in light intensity against per cent reduction in oxygen consumption for various cyanide concentrations, Eymers & van Schouwenburg (2) obtained at low light intensities a straight line relation between light intensity and oxygen consumption. Extrapolated to 100 per cent light intensity the line cut the oxygen consumption at 22 per cent which was taken as the per cent of the oxygen consumption used in luminescence in absence of cyanide, and called the "light respiration." The validity of conclusions drawn from this method of plotting are very doubtful. Such a high percentage of oxygen used in luminescence appears improbable and later van Schouwenburg included in the term "light respiration" not only the oxygen consumed by the luciferin being oxidized and producing light but also the oxygen consumed by the nonluminous oxidation of luciferin. The term "luciferin respiration" would be more appropriate for indicating all the hydrogen transported to oxygen through luciferin, whether light is produced or not.

The small respiration remaining after luminescence has been completely inhibited by cyanide has been called the "rest respiration" and that inhibited by cyanide without reduction of light intensity, the "hemin respiration." The scheme is given on page 548.

Van Schouwenburg found that as the oxygen tension was increased, the part played by the hemin system became less and less, and with 100 per cent oxygen, there was a linear relation between per cent inhibition of oxygen consumption ánd per cent inhibition of luminescence. That is, all concentrations of cyanide which inhibit oxygen consumption also inhibit luminescence. Light emission was relatively much more inhibited at higher oxygen tensions. Van Schouwenburg has interpreted this to mean that the ferment-hemin system is completely paralyzed by cyanide in pure oxygen and the respiration of the bacteria shifted to oxidations making use of the luciferin system as a hydrogen acceptor. The work of Johnson, van Schouwenburg & van der Burg, which has shown that cyanide has no effect whatever on the flash of luminescence after bacteria are deprived of oxygen, has given the final proof that cyanide acts on their constant luminescence in some secondary manner and not on the luciferase itself.

As cyanide has no effect on anaerobic metabolism, it is to be expected that it could exert no influence on the anaerobic redox potential of luminous bacteria, and none was found by Korr (2), who has made a careful and detailed study of various other respiratory poisons.

The results indicate that if respiration and luminescence are affected, the anaerobic redox potential will also change, but not if the respiration and luminescence are unaffected.

Carbon monoxide.—Little work has been carried out to determine the effect of carbon monoxide on luminescence of luminous animals. Only luminous bacteria have been studied and Claren observed no more effect on respiration and luminescence in 95 per cent carbon monoxide than in the corresponding high concentration of nitrogen.[3]

Azide.—Azide has been found by Giese & Fisher (unpublished work) to have an equal inhibiting effect on both respiration and luminescence of bacteria in a glucose medium but only in relatively high (0.01 M) concentrations. In peptone the inhibition appears at much lower concentrations.

Arsenite and iodoacetate.—Both arsenite and iodoacetate greatly reduce the general respiration and luminescence in small concentrations. Bromoacetate is about half as effective as iodoacetate and chloroacetate has no effect [Korr (2)].

Fluoride and pyrophosphate.—On the other hand, fluoride and pyrophosphate do not inhibit respiration and luminescence even in high concentration [Korr (2)]. None of the four last-named inhibitors affect the light of *Cypridina*. They have not been tried, however, on the purified material.

α-Methylglycoside.—An unusual inhibitory effect on the oxidation of certain sugars is produced by α-methylglucoside [Johnson (2, 4, 5)]. The action is not connected with the splitting off of methyl alcohol but presumably is due to competition for the surface of the oxidative catalysts, i.e., α-methylglucoside is adsorbed at the expense of the sugar. Consequently no oxidation of the latter can occur. Lu-

[3] In a recent paper, received in this country since the manuscript on bioluminescence was mailed for publication, van Schouwenburg & van der Burg have made a careful study of the influence of carbon monoxide on *Photobacterium phosphoreum*. The respiration is inhibited by carbon monoxide under all conditions, but is partially reversed by strong light. The luminescence is increased in carbon monoxide–oxygen mixtures whose volume ratio is less than 14, decreased if the ratio is greater than 14. At $CO/O_2 = 7$, the increase in luminescence is 30 per cent. Illumination reduces the increased luminescence at low carbon monoxide–oxygen ratios and also reduces the inhibition at high carbon monoxide–oxygen ratios. The action of carbon monoxide on luminescence is a secondary effect, due to the action of the poison on respiratory oxidations and can be explained by the scheme of Johnson, van Schouwenburg & van der Burg (see p. 548).

minescence is also affected by this specific inhibitor. Later there is an escape from inhibition the meaning of which is not clear.

LUMINESCENCE MECHANISMS

The fundamental mechanism of light production in chemiluminescence is the formation of an excited molecule whose excess energy is emitted as a quantum of light. In the luminescence of *Cypridina* the oxyluciferin or oxidized luciferin molecules may first contain the excess energy which is transferred to luciferase molecules by collision, a case of sensitized chemiluminescence. The excited luciferase molecules then emit the light. Luciferase thus appears to supply excited molecules in addition to acting as catalyst for oxidation of luciferin. The luciferin might act as a reversible hydrogen carrier and could take part in cell respirations. Harvey's (1) scheme for *Cypridina* follows:

Luciferin $(LH_2) + 1/2 O_2 =$ oxyluciferin $(L') + H_2O$
$L' +$ luciferase $(A) = L + A'$
$A' = A + h\nu.$
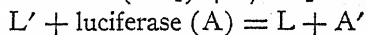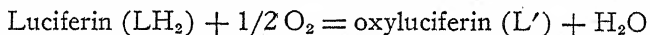
In these equations a prime indicates that the molecule is in an excited state.

Van Schouwenburg looked upon the luminescence of bacteria as intimately bound up with the cell respiration and luciferin as part of the hydrogen transport system, thus:

$$\text{substrate} \rightarrow \text{dehydrogenation system} \underset{\longrightarrow}{\overset{\uparrow}{=\!=}} \begin{array}{l} \text{luciferin} \rightarrow \text{luciferase} \rightarrow \text{III} \\ \vert \rightarrow \text{II} \\ \rightarrow \text{ferment-hemin} \quad \rightarrow \text{I} \end{array}$$
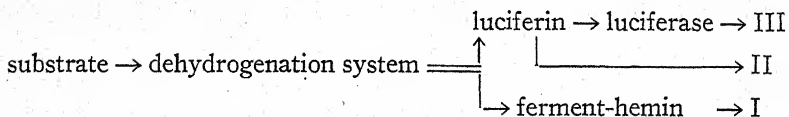
I is the hemin respiration and II and III the luciferin or "light" respiration, although luminescence would only accompany III. The "rest" respiration is not indicated (see p. 546).

A more detailed scheme was published by Johnson, van Schouwenburg & van der Burg to explain their findings in an exhaustive study of the flash of luminescence when oxygen is admitted after anaerobiosis.

(A) L(reversibly oxidized luciferin) $+ XH_2$(substrate) $\rightleftharpoons LH_2$(luciferin) $+ X$
(B) A(luciferase) $+ LH_2 \rightleftharpoons A \cdot LH_2$(luciferin-luciferase combination)
(C) $A \cdot LH_2 + 1/2 O_2 \rightarrow A + L_1$(irreversibly oxidized luciferin) $+ H_2O + h\nu$
(D) $A \rightleftharpoons nB$ (proteolytic products)
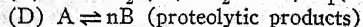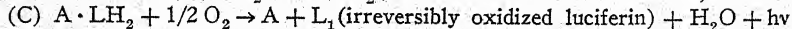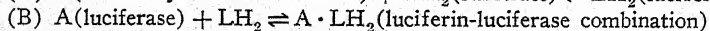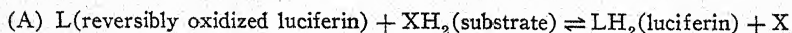
A question arises as to the origin of the oxidized luciferin of equation A. Nothing is known of the precursor of the luciferin-oxidized

luciferin system, although it appears certain that neither nitrogen, sulfur, nor halogen is present in luciferin.[4] Johnson (6) has made a careful study of well-washed suspensions of several species of luminous bacteria and has found that only a certain definite total luminescence can be obtained from them. These bacteria apparently contain a definite amount of luciferin-forming material or of luciferin which can be irreversibly oxidized with luminescence in the presence of a carbohydrate substrate like glucose or fructose. The results in bacteria are best explained on the hypothesis of an irreversible luminescent oxidation of luciferin by luciferase, following reduction of a reversibly oxidized form of luciferin acting as a hydrogen acceptor in respiration.

The digestion of A in equation D by proteolytic enzymes occurs when bacteria have been kept in absence of oxygen for considerable time periods.

The scheme of Chance, Harvey, Johnson & Millikan has already been referred to on page 539.

Spectral Distribution

In 1925 the author studied the quantum efficiency of luminescence of bacteria by measuring the molecules of oxygen absorbed and the quanta of light produced, finding one quantum of light for every 168 molecules of oxygen absorbed. Eymers & van Schouwenburg (2) by more refined methods showed that the quantum efficiency varied with the temperature. At $9.1°$ C., 800 molecules, at $16.1°$ C., 450 molecules, and at $22°$ C., 195 molecules of oxygen are consumed for the emission of one quantum.

Actually the quanta in any animal luminescence vary, since animal light gives a short continuous spectrum with maxima in different regions of the visible. Recent investigations of luminescent spectra have been made by Eymers & van Schouwenburg (1, 3), who studied the spectral energy distribution in the light of several species of bacteria, in *Cypridina,* and also in a number of chemiluminescences, plotting the energy both as a function of wave length and of frequency. Frequency is defined as the reciprocal of wave length expressed in cm., $v = 1/\lambda$. To obtain actual frequencies the figures must be multiplied by the velocity of light, 3×10^{10} cm. per second. The latter method of plotting brings out a few fundamental frequencies whose symmetrical broadening and combination gives rise to the complete curve.

[4] Unpublished observations of Ballentine, Chakravarty & Chase.

Certain frequencies may be common to a number of luminescences, as for example, 18,200 cm.$^{-1}$ which is found in luminous bacteria, in *Cypridina,* in the chemiluminescence and fluorescence of dimethyl-diacridylium nitrate and in the fluorescence of lactoflavin and a fluo-present pigment produced by *Pseudomonas putida.* The lactoflavin fluorescence is made up of one symmetrically broadened frequency, 18,200 cm.$^{-1}$ This strongly supports the validity of an analysis of unsymmetrical spectra as combinations of symmetrical ones. Wherever fundamental frequencies are found, a common configuration of the emitting molecule is to be expected. Takase's analysis of the light of symbiotic bacteria from the deep-sea fish, *Coelorhynchus,* showed fundamental frequencies at 21,200 cm.$^{-1}$ and 19,200 cm.$^{-1}$ On a cane sugar medium the 21,200 cm.$^{-1}$ is the more intense while the reverse is true on a salt medium. Eymers & van Schouwenburg's (1) bacterial spectrograms for *Photobacterium phosphoreum, Ph. splendidum* and a fresh-water *Vibrio* all showed fundamental frequencies at 18,300 cm.$^{-1}$ and 20,400 cm.$^{-1}$ Further data will be necessary to establish the validity of these results.

Akabane has studied the spectral distribution of light from symbiotic bacteria of deep-sea fish of the family Macrouridae and Buck (6) has compared visually or photographically the spectrum of the light of thirteen species of fireflies from Jamaica, B.W.I.

LITERATURE CITED

AKABANE, T., *Japan. J. Physiol.,* **3,** 310–17 (1938)

ALEXANDER, G., *Science,* **82,** 440 (1935)

ANDERSON, R. S., (1), *J. Gen. Physiol.,* **19,** 301–5 (1935)

ANDERSON, R. S., (2), *J. Cellular Comp. Physiol.,* **8,** 251–76 (1936)

ANDERSON, R. S., (3), *J. Am. Chem. Soc.,* **59,** 2115–17 (1937)

BACKOVSKY, J. M., KOMAREK, J., AND WENIG, K., *Věstník Cs. Zoöl. Společnosti Praze,* **7,** 10 pp. (1939)

BOSE, S. R., *Trans. Brit. Mycol. Soc.,* **19,** 97–101 (1935)

BOTHE, F., *Arch. Protistenk.,* **85,** 369–83 (1935)

BROOKS, G., *Compt. rend.,* **210,** 228–30 (1940)

BUCK, J. B., (1), *Science,* **81,** 339–40 (1935)

BUCK, J. B., (2), *Physiol. Zoöl.,* **10,** 45–58 (1937)

BUCK, J. B., (3), *Physiol. Zoöl.,* **10,** 412–19 (1937)

BUCK, J. B., (4), *Nature,* **139,** 801 (1937)

BUCK, J. B., (5), *Quart. Rev. Biol.,* **13,** 301–14 (1938)

BUCK, J. B., (6), *Proc. Rochester Acad. Sci.* (In press)

BUKATSCH, F., *Sitzber. Akad. Wiss. Wien Math. naturw. Klasse, Abt. I,* **145,** 259–76 (1936); *Chem. Ztg.,* **61,** 309 (1937)

CHANCE, B., HARVEY, E. N., JOHNSON, F., AND MILLIKAN, G., *J. Cellular Comp. Physiol.*, **15**, 195–215 (1940)

CHASE, A. M., *J. Cellular Comp. Physiol.*, **15**, 159–72 (1940)

CHASE, A. M., AND GIESE, A. C., *J. Cellular Comp. Physiol.*, **16**, 323–40 (1941)

CLAREN, O. B., *Ann.*, **535**, 122–49 (1938)

DeLERMA, B., *Riv. fis. mat. sci. nat.*, **11**, 615–22 (1937)

DOUDOROFF, M., *Enzymologia*, **5**, 239–43 (1938)

EMERSON, G. A., *Proc. Soc. Exptl. Biol. Med.*, **33**, 36–40 (1935)

EYMERS, J. G., AND VAN SCHOUWENBURG, K. L., (1), *Enzymologia*, **1**, 107–19 (1936)

EYMERS, J. G., AND VAN SCHOUWENBURG, K. L., (2), *Enzymologia*, **1**, 328–40 (1937)

EYMERS, J. G., AND VAN SCHOUWENBURG, K. L., (3), *Enzymologia*, **3**, 235–41 (1937)

FISHER, K. C., AND STERN, J., *Biol. Bull.*, **79**, 342 (1940)

FUJIWARA, T., *J. Sci. Hiroshima Univ.*, **3**, 185–92 (1935)

FULTON, B. B., *J. Elisha Mitchell Sci. Soc.*, **55**, 289–93 (1939)

GIESE, A. C., *Biol. Bull.*, **79**, 346 (1940); *J. Cellular Comp. Physiol.* (In press)

GIESE, A. C., AND CHASE, A. M., *J. Cellular Comp. Physiol.*, **16**, 237–46 (1940)

HANNA, W. F., *Mycologia*, **30**, 379–84 (1938)

HARVEY, E. N., (1), *Ergeb. Enzymforsch.*, **4**, 365–79 (1935)

HARVEY, E. N., (2), *Cold Spring Harbor Symposia Quant. Biol.*, **3**, 261–65 (1935)

HARVEY, E. N., (3), *Trans. Faraday Soc.*, **35**, 233–35 (1939)

HARVEY, E. N., (4), *Living Light*, 328 pp. (Princeton University Press, Princeton, New Jersey, 1940)

HARVEY, E. N., AND KORR, I. M., *J. Cellular Comp. Physiol.*, **12**, 319–23 (1938)

HASAMA, B., *Protoplasma*, **33**, 103–9 (1939)

HEIDT, K., *Biol. Zentr.*, **56**, 100–9 (1936)

HSU, H. L., *Sei-i-kai Med. J.*, **56**, 1–11 (1937)

JOHNSON, F. H., (1), *Science*, **81**, 620–21 (1935)

JOHNSON, F. H., (2), *J. Cellular Comp. Physiol.*, **8**, 439–63 (1936)

JOHNSON, F. H., (3), *Proc. Soc. Exptl. Biol. Med.*, **36**, 387–90 (1937)

JOHNSON, F. H., (4), *J. Cellular Comp. Physiol.*, **9**, 199–206 (1937)

JOHNSON, F. H., (5), *J. Cellular Comp. Physiol.*, **12**, 281–94 (1938)

JOHNSON, F. H., (6), *Enzymologia*, **7**, 72–81 (1939)

JOHNSON, F. H., AND ANDERSON, R. S., *J. Cellular Comp. Physiol.*, **12**, 273–80 (1938)

JOHNSON, F. H., AND CHAMBERS, E. L., *J. Cellular Comp. Physiol.*, **13**, 263–67 (1939)

JOHNSON, F. H., AND HARVEY, E. N., (1), *J. Cellular Comp. Physiol.*, **9**, 363–80 (1937)

JOHNSON, F. H., AND HARVEY, E. N., (2), *J. Cellular Comp. Physiol.*, **11**, 213–32 (1938)

JOHNSON, F. H., AND SHUNK, I. V., *J. Bact.*, **31**, 585–92 (1936)

JOHNSON, F. H., AND VAN SCHOUWENBURG, K. L., *Nature*, **144**, 634 (1939)

JOHNSON, F. H., VAN SCHOUWENBURG, K. L., AND VAN DER BURG, A., *Enzymologia*, **7**, 195–224 (1939)

KANDA, S., *Biol. Bull.,* **77**, 166–73 (1939)

KATO, K., *Japan. J. Zoöl.,* **8**, 251–54 (1939)

KNIPP, C. T., *Science,* **89**, 386–87 (1939)

KOMAREK, J., AND WENIG, J., *Věstník Cs. Společnosti Náuk,* **12**, 1–12 (1938)

KORR, I. M., (1), *Biol. Bull.,* **68**, 347–54 (1935)

KORR, I. M., (2), *J. Cellular Comp. Physiol.,* **6**, 181–216 (1935)

KORR, I. M., (3), *J. Am. Chem. Soc.,* **58**, 1060 (1936)

MACRAE, R., *Nature,* **139**, 674 (1937)

MALUF, N. S. R., (1), *Ann. Entomol. Soc. Am.,* **31**, 374–80 (1938)

MALUF, N. S. R., (2), *Smithsonian Inst. Year Book, Ann. Rept.,* 377–404 (1938)

MILLER, G., *Science,* **81**, 590–91 (1935)

NAKAMURA, H., *Acta Phytochim. (Japan),* **11**, 159–66 (1939)

OKADA, Y. K., *Mem. Coll. Sci. Kyoto Imp. Univ., B,* **10**, 209–28 (1935)

PARKER, H. W., *J. Linnean Soc.,* **40**, 658–60 (1939)

RERABEK, J., AND HYKESOVA, D. E., *Protoplasma,* **28**, 360–64 (1937)

RUEDEMANN, R., *Science,* **86**, 222–23 (1937)

SCHOEPFLE, G. M., (1), *J. Cellular Comp. Physiol.,* **16**, 341–60 (1940)

SCHOEPFLE, G. M., (2), *J. Cellular Comp. Physiol.,* **17**, 106–9 (1941)

SMITH, H. M., *Science,* **82**, 151–52 (1935)

STIER, A., *Z. Morphol. Ökol. Tierre, Abt. A, Z. wiss. Biol.,* **33**, 582–632(1938)

TAKAGI, S., *Annot. Zoöl. Japan,* **15**, 344–49 (1936)

TAKASE, M., *Sei-i-kai-zassi,* **58**, 54–80 (1939)

TAYLOR, G. W., *J. Cellular Comp. Physiol.,* **7**, 409–15 (1936)

VAN SCHOUWENBURG, K. L., (1), *Thesis* (Delft, 1938)

VAN SCHOUWENBURG, K. L., (2), *Enzymologia,* **8**, 344–52 (1940)

VAN SCHOUWENBURG, K. L., AND EYMERS, J. G., *Nature,* **138**, 245 (1936)

VAN SCHOUWENBURG, K. L., AND VAN DER BURG, A., *Enzymologia,* **9**, 34–42 (1940)

WOOD, R. W., *Science,* **90**, 233–34 (1939)

ZIRPOLO, G., *Boll. Zoöl.,* **9**, 49–55 (1938)

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THE CHEMISTRY AND METABOLISM OF BACTERIA

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Because of limitations of space, this review does not attempt to cover the entire subject indicated by the title. Topics omitted include biological nitrogen fixation, polysaccharides, proteins, pigments and other cellular constituents, hydrolytic enzymes, and the metabolism of chemo-autotrophic bacteria. This review is intended to cover the period from December, 1937, to November, 1940; however, many European journals were available only up to about July, 1940.

Nutrient Requirements of Bacteria

Comprehensive reviews of the literature (up to 1938) on growth factors of bacteria and other microorganisms have been published by Lwoff and by Koser & Saunders.

Gram positive facultative anaerobic bacteria.—Though the nutritional requirements of lactic acid bacteria are complex and as yet only imperfectly understood, considerable progress has been made recently, particularly in the study of growth factors. It will be convenient to consider papers dealing with hemolytic streptococci separately.

Following a number of only partially successful attempts to fractionate complex media (Hutner; Eagles, Okulitch & Kadzielawa), several growth factors for lactic acid bacteria, besides riboflavin, have been identified. Snell, Strong & Peterson[1] had earlier obtained highly active concentrates of a substance essential for the growth of thirteen species of lactic acid bacteria. The same authors [Snell, Strong & Peterson (1, 2)] have since shown that the active constituent is pantothenic acid. All organisms of this group so far tested have been found to require this substance or closely related compounds such as hydroxypantothenic acid which is 1.5 to 23 per cent as effective (Mitchell, Snell & Williams). Hydroxypantothenic acid is a synthetic product not known to occur naturally. A biological assay method for pantothenic acid has been developed by Pennington, Snell & Williams which makes use of the growth response of *Lb. helveticus* in a complex basal medium freed of pantothenic acid by alkaline hydrolysis.

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[1] *Ann. Rev. Biochem.,* **7,** 493 (1938).

Recoveries of 94 to 110 per cent are reported. The same organism has been used by Snell & Strong (1) in the assay of riboflavin.

Other known growth factors are needed only by certain species of lactic acid bacteria. Snell, Strong & Peterson (2) have shown that two species of *Lactobacillus* (*Lb. helveticus* and *Lb. arabinosus*) require or are greatly stimulated by nicotinic acid. An improvement in growth due to additions of nicotinic acid or its amide has also been reported by Möller (2) and Orla-Jensen & Snog-Kjaer. Vitamin B_6 has been identified as a growth factor for several species of rod-shaped lactic acid bacteria by Möller (1). The optimum concentration of vitamin B_6 for *Streptobacterium plantarum* (*B. acetylcholini*) is about 0.5 to 1.02 μg. per cc. Its methyl ester is inactive.

Further work upon the riboflavin requirements of lactic acid bacteria has shown that the majority of species tested (seven out of eleven) do not need this substance [Snell & Strong (2)]. Since Schütz & Theorell detected riboflavin in all lactic acid bacteria examined, it appeared probable that species not requiring riboflavin can synthesize it. This was confirmed for *Lb. pentosus, B. brassicae, L. mesenteroides,* and *S. lactis* by both biological and chemical methods. Snell & Strong have also tested the response of *Lb. casei* and *B. lactis acidi* to a number of synthetic flavins and found that it parallels the effects of these same flavins on the growth of rats.

In addition to the known growth factors for lactic acid bacteria (riboflavin, pantothenic acid, nicotinic acid, and B_6), there are other as yet unidentified substances in extracts of yeast, liver, and similar materials which are required for growth. Snell & Peterson studied two such substances acting upon *Lb. helveticus.* One, which is strongly adsorbed by norite, has properties which suggest it to be a purine derivative. The purest preparation gave a half maximum stimulation of growth at a concentration of 0.055 μg. per cc. The second substance has not been extensively studied. Biotin concentrates [Möller (2)] and a thermolabile substance present in malt sprouts (Pan, Peterson & Johnson) have also been reported to stimulate the growth of lactic acid bacteria.

Möller (2) has made some observations on the amino acid requirements of *Streptob. plantarum.* This organism requires glutamic acid, leucine, aspartic acid, and valine, and growth is further improved by cysteine and some other amino acids. A beneficial influence of cystine or cysteine for lactic acid bacteria is also reported by Hutner.

Hemolytic streptococci.—The nutrition of hemolytic streptococci

has now been rather extensively studied. The growth substances shown to be either essential or beneficial include pantothenic acid, B_6, riboflavin, biotin, thiamin, nicotinic acid, glutamine, and adenine or certain other purines. The necessity for pantothenic acid was first shown by Woolley & Hutchings (1) with *S. epidemicus* and has since been confirmed in other laboratories with other species [McIlwain (1); Subbarow & Rane; Pappenheimer & Hottle]. However, at least some strains can use the acid fragment of pantothenic acid in place of the whole molecule (Woolley). Woolley & Hutchings (1) also found that riboflavin and a nonspecific reducing agent such as hydrogen sulfide, thioglycolic acid, or reduced iron, are essential for the growth of some strains; others do not require riboflavin (Krauskopf, Snell & McCoy).

Glutamine has been identified by McIlwain, Fildes, Gladstone & Knight [see also Landy (2)] as the active substance in meat extracts which are required for the growth of freshly isolated strains of *S. hemolyticus*. After several subcultures under laboratory conditions, glutamine is no longer required presumably because the organisms have acquired the ability to synthesize it. Group A hemolytic streptococci appear to be much more dependent upon an outside source of glutamine than are organisms of other groups; out of ten group B strains only two were stimulated by glutamine, none required it (Fildes & Gladstone). As little as 0.05 µg. per cc. of glutamine will allow growth of fastidious strains; it is remarkable that the yield of cells appears to be independent of the quantity of added glutamine. The specificity of glutamine appears to be very great since McIlwain (2) has tested a considerable number of related compounds with entirely negative results. No direct evidence is at present available concerning the function of glutamine.

Besides the growth factors mentioned above, hemolytic streptococci are known to require a number of amino acids and other substances usually supplied in protein hydrolyzates. Several more or less successful attempts have been made to replace these complex substrates by completely synthetic media. Hutchings & Woolley and Woolley & Hutchings (2) obtained normal growth of *S. zymogenes* and *S. mastitides* (Lancefield groups D and B) in a medium containing nineteen amino acids in addition to riboflavin, pantothenic acid, and B_6. Only two of the amino acids, glutamic acid and tryptophane, were actually shown to be indispensable. Although the others are not absolutely essential they greatly increase growth. Cystine, the only

sulfur-containing amino acid used, could be replaced by either methionine or hydrogen sulfide. Streptococci of the Lancefield A, C, E, and F groups do not grow in the above-mentioned medium.

McIlwain (3) was able to obtain good growth of a strain of *S. hemolyticus* in a more complex synthetic medium containing some forty amino acids and other compounds including pantothenic acid, B_6, glutamine, thiamin, riboflavin, nicotinic acid, pimelic acid, glucosamine, betaine, thiochrome, purines, and mercaptoacetate. Several of these constituents had previously been shown by Rane & Subbarow (1) to be beneficial. Perhaps the most interesting result of McIlwain's study is the demonstration that different constituents of the synthetic medium show different degrees of essentiality. Some constituents appear to be absolutely essential for growth, others are replaceable by compounds of similar structure, while still others can be substituted by compounds entirely unrelated in structure or chemical properties. A striking example of the last-mentioned group of constituents is provided by the work of Pappenheimer & Hottle on the nutrition of a group A strain of hemolytic streptococcus. This organism could be grown in a semisynthetic medium containing many of the constituents used by McIlwain and by Hutchings & Woolley. In addition it was shown that thiamin, nicotinic acid, adenine or related purines, tyrosine, and probably biotin are essential for growth. Of particular interest is the relation between the purine and the carbon dioxide requirements. Adenylic acid (or certain other purines) appears to be essential only when the carbon dioxide tension is low (0 to 2 mm. Hg) ; at higher carbon dioxide tensions (20 mm. Hg) only a slightly beneficial effect of adenylic acid could be observed.

Highly virulent pneumococci of Types II, V, and VIII have been shown by Rane & Subbarow (2) to grow well in a synthetic medium containing fifteen amino acids, glucose, flavin, thioglycolic acid, choline, pantothenic acid, and nicotinic acid. Omission of any of the last three compounds mentioned prevents growth.

Several years ago the nutritional requirements of *Staph. aureus* appeared to have been largely solved by the demonstration that the organism can grow in an amino acid or ammonia nitrogen medium supplemented with thiamin and nicotinic acid. Further work has shown, however, that the growth of many strains in this medium is relatively poor and can be very greatly improved by the addition of plant or animal extracts. The principal active constituent of such extracts is evidently biotin, which Kögl & van Wagtendonk (see also

Porter & Pelczar) have found to give maximum stimulation (500 to 2000 per cent) at a concentration of about 0.005 µg. per cc. It is significant that in the presence of biotin the quantities of thiamin and nicotinic acid required for maximum growth are greatly reduced. The specificity of thiamin and nicotinamide as growth factors for staphylococci has been further emphasized by the study of related compounds [Knight & McIlwain; Landy (1)].

The nutritional requirements of propionic acid bacteria appear to be simpler than those of lactic acid bacteria. Wood, Anderson & Werkman have found that several *Propionibacterium* species require an acid-ether extract of yeast which is not replaceable by a mixture of nicotinic acid, thiamin, pimelic acid, uracil, β-alanine, and pantothenic acid; however, Snell, Strong & Peterson (2), using *P. pentosaceum*, were able to substitute pantothenic acid for the yeast factor. The cause of this discrepancy is not clear. Amino acids, riboflavin, and thiamin are generally beneficial but can be dispensed with by many strains after "training." Trained cells satisfy their thiamin requirements by synthesis [Silverman & Werkman (1)].

Corynebacterium diphtheriae has been known for some time[2] to be able to grow on a modified hydrolyzed casein medium supplemented with pimelic acid, nicotinic acid, and β-alanine. The casein hydrolyzate has been shown by Mueller (1); Evans, Handley & Happold; and Evans, Happold & Handley to be replaceable by a mixture of amino acids. For most strains tryptophane, cystine, histidine, phenyl alanine, methionine, glycine, valine, and glutamic acid are satisfactory, though some exacting intermediate strains appear to require additional amino acids. Growth is also improved by additions of manganese, copper, and zinc; Mueller's experiments provide the most clear-cut example to date of the importance of these elements for bacteria. β-Alanine may be replaced by either carnosine [Mueller (2)] or pantothenic acid (Mueller & Klotz; Evans, Handley & Happold); *gravis* strains require the latter. Since pantothenic acid stimulates growth more quickly and in lower concentrations than does β-alanine, it seems probable that β-alanine is effective only following the synthesis of pantothenic acid. Evans, *et al.* have shown that nonexacting strains, growing in the presence of β-alanine, do in fact synthesize pantothenic acid as well as thiamin, riboflavin, and coenzyme I or II.

Gram negative facultative anaerobic bacteria.—A few strains of

[2] *Ann. Rev. Biochem.*, 7, 492 (1938).

the dysentery bacillus can grow in a synthetic amino acid medium without added growth factors, but most strains require nicotinic acid or closely related compounds [Dorfman, Koser & Saunders (1); Dorfman, Koser, Reames, Swingle & Saunders; Dorfman, Koser, Horwitt, Berkman & Saunders]. The growth-promoting activities of twenty-five pyridine derivatives for the dysentery organism closely parallel their activities for staphylococci and animals. Nicotinamide is about ten times as effective as nicotinic acid or the pyridine coenzymes when a comparison is made with cultures incubated for a short time. This is taken to indicate that nicotinic amide does not serve solely for the formation of coenzymes; it seems possible, however, that the observed results can be equally well explained on the basis of permeability effects. By means of biological assay methods the dysentery bacillus, growing in a medium containing amino acids and nicotinic acid, is shown to synthesize coenzyme I or II, riboflavin, thiamin, and possibly biotin. Kligler & Grosowitz could observe no improvement in the growth of *E. typhosum, E. coli,* or *Salm. paratyphi B* as a result of the addition of nicotinic acid to a peptone medium, though both *Salm. paratyphi A* and *Sh. dysenteriae* were stimulated.

Proteus vulgaris and closely related species also generally require nicotinic acid or one of its derivatives as a sole growth factor, though some strains grow well on a simple ammonium lactate medium [Fildes (1); Pelczar & Porter (1); Lwoff & Querido (2)]. During growth nicotinic acid is converted at least in part into coenzyme I or II. Lwoff & Querido (1) have made use of this organism for the biological assay of nicotinic acid and related compounds. Not all *Proteus* species have equally simple nutritional requirements. In addition to nicotinic acid, *P. morganii* needs other nitrogenous organic nutrients including pantothenic acid [Pelczar & Porter (2)].

Some strains of typhoid bacillus are known to grow on a glucose-ammonia nitrogen medium while others appear to require tryptophane. Burrows (1, 2) has shown that tryptophane is not always essential since with at least one strain, it can be replaced by lysine; another strain which does not grow even with tryptophane, develops normally in the presence of arginine or glutamic acid. Furthermore, organisms that require tryptophane to initiate growth are nevertheless able to synthesize the amino acid in significant amounts. The main effect of tryptophane upon growth is to shorten the lag phase; the yield of cells is largely independent of the quantity of tryptophane added. Hydrogen sulfide, used as a sulfur source, affects growth in the same

manner while the quantity of available ammonia nitrogen controls the cell yield without altering the length of the lag phase. The influence of tryptophane on the typhoid bacillus is similar to that of glutamine on hemolytic streptococci (Fildes & Gladstone).

Other bacteria.—Earlier controversies concerning the growth factor nutrition of nodule bacteria have been largely resolved. It is now generally agreed that most strains of *Rh. trifolii, Rh. leguminosarum, Rh. meliloti,* and *Rh. phaseoli* require or are greatly stimulated by various plant and animal extracts containing a heat-stable substance which has been referred to as "coenzyme R" [Allison & Minor (1); Nilsson, Bjälfve & Burström (1); West & Wilson (1)]. There are, however, a few strains belonging to the above species, as well as organisms of the cowpea, soybean, and lupine groups, that grow well in a mineral salts–sugar medium and respond only slightly if at all to addition of coenzyme R preparations. Several such organisms have been shown by Allison & Minor (2) to synthesize coenzyme R and to liberate it into the culture medium.

The chemical nature of coenzyme R is of particular interest. West & Wilson (2), using both chemical and biological methods, obtained abundant evidence indicating the identity of coenzyme R with biotin. Final proof of the importance of biotin for rhizobia was provided by Nilsson, Bjälfve & Burström (2) who showed that the addition of the pure compound (0.0005 µg. per cc.) to a suitable synthetic medium allows excellent growth of many strains. That biotin is not the only growth substance needed by nodule bacteria is evident from the work of West & Wilson (1). These authors found that the initiation of growth in a mineral salts–sugar medium is dependent upon the presence of a thermolabile factor which is synthesized by the bacteria and is ordinarily provided in adequate amounts by the inoculum. This thermolabile factor is replaceable by either thiamin or riboflavin in suitable concentrations; since these substances are also shown to be synthesized by the organism it seems probable that they are the active constituents of the thermolabile growth-initiating factor. The necessity of thiamin for some strains of *Rhizobium* has been demonstrated independently by Bjälfve, Nilsson & Burström. β-Alanine [West & Wilson (2)] appears to exert a slight beneficial effect, while amino acids generally do not stimulate growth.

The bacteria belonging to the genus *Clostridium* clearly have very diverse nutritional requirements. At least one species, *Cl. pasteurianum,* can develop in a medium free from nitrogen compounds; other

species, like *Cl. sporogenes* and *Cl. parabotulinum* (Elberg & Meyer), require a variety of amino acids and one or more growth factors. At least some of the species which produce butyl alcohol appear to occupy a position between these extremes. *Cl. butylicum* has been shown by Snell & Williams to grow luxuriantly on a glucose-salts-asparagine medium following the addition of biotin. Previously an unidentified acidic substance, extractable from cereal grain, liver, and similar sources, had been found to allow growth of several clostridia, including *Cl. butylicum,* in the same basal medium (Woolley, McDaniel & Peterson; McDaniel, Woolley & Peterson; Brown, Wood & Werkman). The relation between this substance and biotin is not yet clear. *Cl. acetobutylicum* also appears to be stimulated by biotin, though the evidence is not conclusive (Weizmann & Rosenfeld). The discovery by Tatum, Peterson & Fred that asparagine and related compounds increase the yield of "solvents" in butyl alcohol fermentation has been confirmed (Brown, Stahly & Werkman); it should be emphasized, however, that adenine and even ammonium sulfate exert a similar though smaller effect.

Further insight into the complexities of amino acid nutrition is given by Gladstone who has shown, using *Bac. anthracis,* that the nutritional value of one amino acid may be greatly influenced by the presence or absence of other amino acids. *Bac. anthracis* can be grown in a chemically defined amino acid medium containing (among others) valine, leucine, and isoleucine. Omission of any one of these acids prevents or greatly diminishes growth, thus indicating their essential nature. However, when all three are simultaneously omitted growth occurs and then the addition of any one of the amino acids is harmful. These results are clarified by the demonstration that definite antagonisms among amino acids exist. For example, the inhibiting effect of valine alone is counteracted by leucine, while the toxicity of isoleucine is overcome only by both valine and leucine. Other antagonisms, between valine and α-amino butyric acid and between threonine and serine were also observed. An explanation of these antagonisms is advanced in terms of a competitive blocking of enzyme surfaces.

Functions of nutrients.—Although much work has been done on the general nutrient requirements of bacteria, relatively few investigations have dealt with the specific functions of individual nutrients. Undoubtedly the most interesting and important results in this field are those concerned with the functions of carbon dioxide which has long been known to be essential for bacterial growth. The demon-

stration by means of isotopic carbon of the conversion of carbon dioxide into acetic, propionic, and succinic acids by typically heterotrophic bacteria is discussed elsewhere in this review. Here attention is called particularly to the formation of various unidentified cell constituents from carbon dioxide by a number of microorganisms (yeast, *E. coli*, *Clostridium*, and *Propionibacterium*) as well as by plant and animal tissues (Ruben & Kamen). This seems to be a very general phenomenon. Hes has presented evidence that carbon dioxide also may be involved as a catalyst in oxidation-reduction reactions. The decolorization of methylene blue by suspensions of several types of bacteria and by yeast maceration juice is prevented or greatly delayed in the absence of carbon dioxide.

The role of thiamin in the metabolism of *Staph. aureus* and of propionic and lactic acid bacteria has been studied by Hills (1) and by Silverman & Werkman (1, 2, 3). See also Lipmann (1). The addition of thiamin to thiamin-deficient cells of these organisms results in a marked increase in the rate of carbon dioxide production from pyruvate; with propionic and lactic acid bacteria, the rates of sugar and lactate breakdown are also somewhat increased. Either thiamin or the constituent thiazole and pyrimidine together are effective for staphylococci. With *Prop. pentosaceum* thiamin is shown to be converted into cocarboxylase.

Dorfman, Koser & Saunders (2) report a stimulation of methylene blue reduction and oxygen uptake by dysentery bacteria resulting from the addition of nicotinic acid or amide to deficient cells. Mention should also be made of the observation of Pratt & Williams that pantothenic acid increases the respiration of living yeast and to a lesser extent stimulates fermentation by yeast juice.

OXIDATIVE METABOLISM

Of considerable theoretical importance is the work of Kempner on the influence of oxygen tension on respiration. Using several types of aerobic and facultatively anaerobic bacteria, as well as animal cells, he has shown that contrary to the widely accepted opinion of Warburg, respiration commonly decreases as the oxygen tension is diminished. This dependence of respiration upon oxygen tension holds for organisms whose respiration is catalyzed by the iron-containing phaeohaemin (*E. coli*) as well as for organisms using the iron-free yellow enzyme (*Pneumococcus*).

Acetic acid bacteria.—The environmental conditions influencing

the oxidative activity of *Acetob. suboxydans* have been further studied[3] by Butlin (1, 2). The previous finding that cells grown in a medium buffered with calcium carbonate oxidize glucose further than do cells grown in an acid medium was extended by showing that a continuous change in oxidizing ability occurs as a culture ages. Cells from young cultures produce carbon dioxide and take up considerably more than one atom of oxygen per molecule of glucose while cells from old cultures produce little or no carbon dioxide and take up only one atom of oxygen. Similar results are reported by Kluyver & Boezaardt (1) who, however, found that the oxygen uptake by old cultures of their strain of *A. suboxydans* is equivalent to two atoms per molecule of glucose, indicating the formation of ketogluconic acid. The above mentioned changes in oxidative activity with age occur much more rapidly in unbuffered than in buffered media due to the greater acidity of the latter. The importance of acidity is proved by the demonstration that the enzyme system responsible for the oxidation of glucose beyond the stage of gluconic acid (Butlin's organism) is preferentially inactivated by treating highly oxidative cells for one half hour with a suitable concentration of hydrochloric acid. The incomplete oxidations characteristic of *A. suboxydans* are evidently in part a consequence of the normally acid environment.

The acidity of the medium appears also to determine the carbon atom at which gluconic acid is oxidized by *A. suboxydans* (Bernhauer & Knobloch). When glucose is oxidized in a moderately acid medium first gluconic acid, then 5-ketogluconic acid is formed. However, when the organism is allowed to act upon calcium or potassium gluconate in a neutral medium, the main product (60 per cent of theoretical) is 2-ketogluconic acid. The oxidation of calcium mannonate appears to yield the same product.

As a result of the study of the oxidations of a number of sugar alcohols (Hann, Tilden & Hudson), the conclusion is reached that *A. suboxydans* is somewhat more specific than *A. xylinum* as to the compounds it can attack. *Meso*-inositol is oxidized almost quantitatively by *A. suboxydans* to inosose (monoketoinositol) [Kluyver & Boezaardt (2)]. Studies of the conversions of perseitol to persulose (Tilden), erythritol to *l*-erythrulose (Whistler & Underkofler), mannitol to levulose (Fulmer, Dunning & Underkofler), α,β-propylene glycol to acetol [Butlin & Wince (2)], glycerol to dihydroxyacetone

[Butlin (3)] and glucose to gluconic acid [Butlin & Wince (1)] are largely of technical interest.

Wieland & Pistor have continued their study of oxidations carried out by *Acetob. peroxidans*. Among other things they show that ethanol is oxidized more rapidly by quinone than by oxygen, the reverse being true for formate oxidation. Evidence that the oxidations of ethanol and formate are catalyzed by distinct dehydrogenase systems is provided by the greater sensitivity of the latter process to inhibition by quinone and oxygen. The reaction between molecular hydrogen and oxygen is also progressively and reversibly inhibited by oxygen when it makes up more than about 10 per cent of the gas phase. At lower partial pressures no inhibition occurs. A similar inhibition of respiration by high oxygen concentration has been observed by Claren, using a luminous bacterium; with this organism also the percentage inhibition varies greatly with the substrate. It is interesting that the aerobic oxidation of ethanol by *A. peroxidans* is only very slightly influenced by carbon monoxide.

A comprehensive review of the biochemical activities of acetic acid bacteria is given by Bernhauer.

Oxidative assimilation.—Clifton & Logan [see also Brewer & Werkman (2)] have found that *E. coli,* like a number of other aerobic microorganisms previously studied, assimilates a definite and large part of the substrates it oxidizes. This oxidative assimilation is preferentially inhibited by suitable concentrations of sodium azide and dinitrophenol, and the substrates are then completely oxidized. These results are shown to apply to growing cultures as well as to cell suspensions.

A thorough investigation of the growth and respiration of *Rh. trifolii* has been carried out by Hoover & Allison. Besides measuring the quantities of substrate (sucrose) and oxygen consumed and carbon dioxide produced in growing cultures, cell synthesis and cell composition were directly determined. The most striking result is the demonstration that the growth rate is proportional to $(Q_{O_2} - K)$ where K is the Q_{O_2} of cells that have ceased to grow; this is further evidence of the close relation between respiration and assimilation already shown by experiments with cell suspensions. The efficiency of substrate utilization is quite high, the economic coefficient of young cultures being 40 to 50. *Bac. glycinophilus* is another example of a bacterium assimilating a large percentage of its substrate in growing cultures (Rippel & Nabel). With ammonium phosphate as a sole

nitrogen source, an average of about 65 per cent of the substrate (glucose) is converted into cell material.

Other oxidative processes.—Klein finds that $l(-)$-aspartic and $l(+)$-glutamic acids are the only amino acids oxidized by cell suspensions of *Hemophilus parainfluenzae*, the reactions being:

$$COOH \cdot CH_2 \cdot CHNH_2 \cdot COOH + O_2 \rightarrow$$
$$2\ CO_2 + NH_3 + CH_3 \cdot COOH$$
$$COOH \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH + 2.5\ O_2 \rightarrow$$
$$3\ CO_2 + NH_3 + CH_3 \cdot COOH.$$
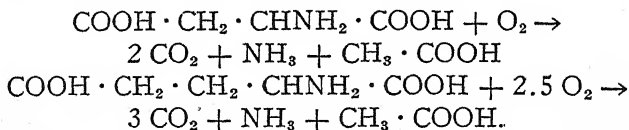
The available evidence indicates that the successive steps in the oxidation of glutamic acid are α-ketoglutarate, succinate, fumarate, malate, oxalacetate, pyruvate, acetaldehyde, and acetate. Most of these compounds (α-ketoglutarate and oxalacetate were not studied) are oxidized with the expected oxygen uptake and carbon dioxide production. Except for the oxidation of succinate to fumarate all these steps appear to require pyridine coenzymes. Egami has identified glyoxylic acid as a product of glycine oxidation by an unidentified organism which uses nitrate as the hydrogen acceptor.

Suspensions of *E. coli* grown in the complete absence of tryptophane are able to oxidize tryptophane to indole [Fildes (2)]. The addition of tryptophane to the growth medium, however, increases the rate of indole formation about twenty-five fold; indole and indole acrylic acid exert a smaller stimulating effect. Growing cultures, but not cell suspensions, form indole from indole acrylic acid and the evidence suggests that tryptophane is an intermediate in this conversion. The study of a large number of tryptophane derivatives (Baker & Happold) further confirms the view that only tryptophane or substances convertible into tryptophane give rise to indole. This leads to the suggestion that indole formation involves a reductive removal of the entire tryptophane side chain in one step.

Krebs' interpretation[4] of pyruvate oxidation by *Gonococcus* and *Staphylococcus* as an anaerobic dismutation followed by a reoxidation of lactate to pyruvate can no longer be accepted in view of the work of Barron & Lyman. The rate of pyruvate dismutation is frequently much less than the rate of oxidation; also hydrogen sulfide reduces the oxidation rate of lactate far more than of pyruvate. A direct oxidation of pyruvate as indicated by Lipmann (1) probably occurs.

Claren, using a luminous bacterium, has uncovered a catalysis of the hydrogen-oxygen reaction by the fumaric-succinic acid system. Carefully washed cell suspensions do not act upon mixtures of hydrogen and oxygen, but a rapid reaction occurs on the addition of either fumaric or succinic acids if the gas phase contains less than 20 per cent oxygen. At higher partial pressures of oxygen the reaction is completely inhibited. The rate of the hydrogen-oxygen reaction is independent of the concentration of added fumarate or succinate over a considerable range; however, with small amounts of either acid the rate soon falls off due to the destruction of the catalytic system by irreversible oxidation.

Various strains of hemolytic streptococci and other lactic acid bacteria are shown to differ considerably with respect to the substrates oxidized and the quantities and rates of oxygen consumption (Barron & Jacobs; Hansen).

BACTERIAL ENZYMES

One reason for the relatively small amount of information available concerning bacterial enzyme systems is the difficulty of obtaining cell-free bacterial enzyme preparations. Booth & Green have developed a very efficient wet crushing mill which has made possible for the first time the preparation of a considerable number of bacterial enzyme systems in a cell-free condition. Wiggert, Silverman, Utter & Werkman have succeeded in liberating enzymes from several species of bacteria by the simpler, though perhaps not always as efficient, method of grinding a paste of cells and powdered glass in a mortar. Attention should also be called to methods involving rapid drying [Lipman (1)], repeated freezing and thawing (Adler, Hellström, Günther & von Euler), and supersonic oscillation (Kocholaty, Smith & Weil) which have been successfully used in the preparation of specific enzyme systems.

Nonnitrogenous substrates.—The malic and formic dehydrogenases obtained by grinding *E. coli* in the Booth-Green mill have been studied in some detail by Gale & Stephenson (2) and Gale (2). When the cell juice is centrifuged the former enzyme remains in the liquid while the formic dehydrogenase separates with the solid phase. The malic dehydrogenase from *E. coli* is shown to require coenzyme I and diaphorase for reaction with methylene blue; when in addition oxygen and cyanide are present, malate is oxidized with the uptake of one atom of oxygen per mol to form oxalacetate. The reaction between

malic acid and coenzyme I in the presence of the enzyme is reversible. The formic dehydrogenase appears not to require coenzyme I or II but reacts with oxygen via cytochrome-b; this latter conclusion is based upon spectroscopic evidence and cyanide inhibition experiments. The enzyme preparations are inactivated by oxygen so rapidly that a constant rate of formate oxidation could only be obtained by using oxygen tensions below atmospheric.

In a series of excellent investigations Lipmann (1, 2) has elucidated the mechanism of pyruvic acid oxidation by *B. delbrückii*. The net reaction is

$$CH_3 \cdot CO \cdot COOH + \tfrac{1}{2} O_2 \rightarrow CH_3 \cdot COOH + CO_2.$$
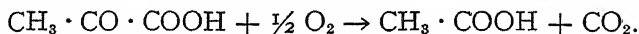
Hydrogen peroxide is the primary product of oxygen reduction but it immediately disappears by reaction with pyruvate. The enzyme system catalyzing the above reaction has been shown (by the use of extracts of dried or acetone-treated cells) to consist of at least five components: thiamin pyrophosphate; flavin adenine dinucleotide; manganous, magnesium, or cobaltous ions; protein; and inorganic phosphate. Most interesting is the role of phosphate. During the oxidation inorganic phosphate disappears and is converted in the presence of adenylic acid into adenosine pyrophosphate. The mechanism of this coupled dehydrogenation and phosphorylation appears to be (a) the formation of a phosphorylated pyruvic acid, (b) the simultaneous dehydrogenation and decarboxylation of this compound to give acetylphosphate and carbon dioxide, and (c) a reaction between acetylphosphate and adenylic acid to give acetic acid and adenosine pyrophosphate. As evidence for this mechanism, reaction (c) has been shown to occur and a phosphate ester having the general properties of acetylphosphate has been observed as an oxidation product of pyruvate in the presence of inorganic phosphate. Enzyme preparations used in the above experiments normally show almost no activity in the absence of oxygen, but when riboflavin or other suitable hydrogen carrier is added a dismutation (involving coupling of the pyruvic and lactic dehydrogenase systems) occurs with the formation of acetic and lactic acids and carbon dioxide.

Katagiri & Kitahara, and Katagiri & Murakami have found a marked specificity in the action of lactic acid bacteria on optically active isomers of lactic and phosphoglyceric acids. Organisms attack the same isomer they produce; species possessing the enzyme racemiase utilize both optical isomers.

Nitrogenous substrates.—Gale (1), and Gale & Stephenson (1) have continued[5] the study of the deaminase of *E. coli.* Deaminations of aspartic acid and serine occur either aerobically or anaerobically; both have a very high Q_{NH_3} (200 to 1000) and are strongly inhibited by glucose in the growth medium. Washed suspensions of cells lose their serine deaminase activity on standing. The fact that inorganic phosphate and reducing agents together prevent the loss of activity and reactivate inactive preparations is taken to indicate that an unidentified coenzyme is involved in the deamination reaction; this may be the adenine-flavin coenzyme shown by Warburg & Christian to be part of the kidney alanine oxidase systems. The deamination of aspartic acid by *E. coli* is caused by two distinct enzymes, aspartase I and II, which were obtained as cell-free preparations. Aspartase II differs from the previously studied aspartase I in chemical properties and also in being activated by adenosine and inhibited by toluene. The products of aspartic acid decomposition by aspartase II appear to be ammonia and fumaric acid, though the preparations studied contained fumarase and consequently formed malic acid. The ratio of the two fumarases in *E. coli* varies with the strain and the cultural conditions.

Stephenson & Trim have studied the action of *E. coli* on adenine and related compounds. Muscle adenylic acid is dephosphorylated and deaminated almost simultaneously, the former process probably preceding the latter. One per cent phenol completely inhibits deamination without affecting the phosphorylation reaction. Adenosine, adenylic acid, and adenine are deaminated at successively slower rates. It is significant that the presence of adenosine increases the rate of ammonia formation from adenine and also that ribose in combination with adenine is fermented about ten times as rapidly as free ribose.

Adler, Hellström, Günther & v. Euler have shown that the reactions involved in the synthesis and breakdown of glutamic acid are the same in *E. coli* as in yeast, animal tissues, and higher plants:

$$COOH \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH + \text{``coenzyme''} \rightleftharpoons$$
$$COOH \cdot CH_2 \cdot CH_2 \cdot C = NH \cdot COOH + \text{``reduced coenzyme''}$$
$$COOH \cdot CH_2 \cdot CH_2 \cdot C = NH \cdot COOH + H_2O \rightleftharpoons$$
$$COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH + NH_3.$$
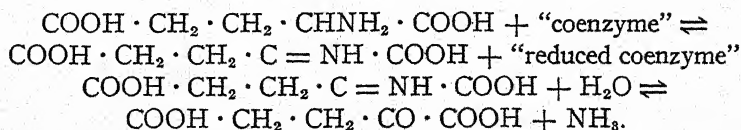
A cell-free preparation of the glutamic acid apodehydrogenase catalyzing the first reaction was obtained by repeated freezing and thaw-

ing, followed by grinding; only coenzyme II would react with this preparation. The second reaction is nonenzymatic. Since the transfer of amino groups from glutamic acid to keto acids is also catalyzed by *E. coli,* the above reactions provide a possible general mechanism for the synthesis of amino acids from ammonia.

Cell suspensions of *E. coli* [Desnuelle & Fromageot; Desnuelle (1, 2); and an anonymous report] decompose cysteine under anaerobic conditions with formation of equimolar quantities of hydrogen sulfide and ammonia by means of an adaptive enzyme, cysteinase. Weak preparations of the enzyme can be obtained from cells by drying or by acetone treatment. Hydrogen sulfide appears to be formed from cystine only after the latter is reduced to cysteine. Glucose is an excellent hydrogen donor for this purpose while hydrogen and formic acid are ineffective or inhibitory. Only the natural isomers, $l(-)$-cysteine and $l(-)$-cystine, are attacked.

The decompositions of cysteine and cystine by *Prop. pentosaceum* appear to involve mechanisms different from those used by *E. coli* (Desnuelle, Wookey & Fromageot). Earlier papers from the same laboratory on cysteine decomposition by *E. coli* should be disregarded (Desnuelle & Fromageot).

A specific and sensitive method for the analysis of creatine and creatinine in tissues has been developed (Miller, Allinson & Baker) by making use of the creatinine oxidizing organism isolated by Dubos & Miller.[6] This organism, now called *Corynebacterium ureafaciens,* is shown to form urea as a result of the oxidation of several other nitrogenous substances including uric acid, allantoin, allantoic acid, and hypoxanthine (Krebs & Eggleston). It should be pointed out that bacterial urea formation from uric acid is not a recent discovery as the authors assume, but was carefully studied a generation ago by Liebert who demonstrated the formation of allantoin as an intermediate. Of considerable interest is the report that *C. ureafaciens,* under anaerobic conditions, is able to convert creatine to creatinine (Dubos & Miller). The adaptive enzyme system concerned was obtained cell-free and was found to have a high substrate specificity.

The decarboxylation of amino acids to form amines has been studied by means of both growing cultures and cell suspensions. Eggerth found histamine to be formed from histidine by a large number of intestinal bacteria. Growth media containing peptone or similar

6 *Ann. Rev. Biochem., 7,* 506 (1938).

complex nitrogenous materials and a carbohydrate are generally more favorable for amine formation than are simpler synthetic media. The carbohydrate is desirable because it increases growth and leads to the establishment of an acid reaction which is generally essential for amine production. However, bacteria grown on acid media without carbohydrate are equally active. The optimum reaction for histamine production by growing cultures of most bacteria is pH 5.0 to 5.5; only certain strains of *A. aerogenes* form the amine above pH 6.5. The most favorable temperature for amine production is generally below the optimum for the growth rate. Gale (3) has shown that cell suspensions of *E. coli* can quantitatively decarboxylate arginine, lysine, histidine, ornithine, and glutamic acid to form agmatine, cadaverine, histamine, putrescine, and γ-amino butyric acid, respectively. Highly active suspensions can be obtained only from young cultures in acid media and the pH-activity curves have sharp maxima in the range pH 4.0 to 5.0 which are characteristic of the amino acids attacked. Because of this and the unequal ability of different strains to attack the various amino acids, it is concluded that a specific enzyme is concerned with the decarboxylation of each acid. One questionable point in this paper is the author's supposition that decarboxylation reactions may provide energy for growth in acid media. *Streptococcus faecalis* is able to convert tyrosine to tyramine [Gale (4)]. The conditions favoring this reaction are very similar to those favoring amine formation by *E. coli*. The substrate specificity of the decarboxylating enzyme is quite evident since besides tyrosine only arginine is attacked and it is decomposed in a different manner with formation of two mols of ammonia, one mol of carbon dioxide, and one mol of ornithine per mol arginine. Gale (5) has also studied formation of putrescine by the action of mixed cultures of *S. faecalis* and *E. coli* on arginine; the yield of putrescine depends upon the pH and the relative quantities of the two organisms.

Hills (2) presents evidence indicating that the ability to convert arginine to ornithine, ammonia, and carbon dioxide by a hydrolytic reaction is characteristic of *Staphylococcus aureus* and *Streptococcus* species. Other amino acids are also attacked by the former (see also Webster) but not by the latter organisms. The formation of ammonia from arginine cannot be due to the combined actions of arginase and urease since the latter enzyme is absent or insufficiently active in these organisms; the active enzyme is called "arginine dihydrolase" to distinguish it from ordinary arginase. A somewhat similar formation of

ammonia from uric acid and other purines by an organism devoid of urease has been reported by Beck.

Tarr has studied the reduction of trimethylamine oxide to trimethylamine by bacteria isolated from spoiled fish. The reduction may be coupled with oxidations by various dehydrogenase systems and under suitable conditions goes to completion. The activating enzyme, trimethylamine-oxidease, is not inhibited by cyanide.

FERMENTATIVE METABOLISM

Propionic acid fermentation.—The complexity of the propionic acid fermentations of glucose, glycerol, and other compounds is becoming increasingly evident. Whereas a few years ago the only products known to be derived from glucose were propionic acid, acetic acid, and carbon dioxide, it is now established that lactic, pyruvic, and succinic acids and propyl alcohol are also commonly formed in smaller amounts [Wood & Werkman (1) ; Phelps, Johnson & Peterson (2)]. The relative quantities of these products are not constant but vary considerably with the time and conditions of incubation. Fromageot & Bost, and Fromageot & Safavi have especially emphasized the importance of assimilatory processes in modifying the proportions of catabolic products; under favorable conditions 5 to 10 per cent of the substrate carbon is converted into cell material.

The remarkable results of Wood & Werkman[7] on the utilization of carbon dioxide by propionic acid bacteria have now been completely confirmed and extended in several laboratories [Wood & Werkman (1); Phelps, Johnson & Peterson (1); Carson & Ruben]. Carbon dioxide uptake can be observed in both growing cultures and cell suspensions with the aid of chemical, physical, or manometric methods. The original observations on carbon dioxide uptake were made with glycerol media. A similar disappearance of carbon dioxide also occurs during fermentations of mannitol, adonitol, erythritol, and rhamnose in an atmosphere of carbon dioxide, while with glucose and other carbohydrates carbon dioxide is evolved. A comparison of the quantities of carbon dioxide evolved in atmospheres of carbon dioxide and nitrogen indicates, however, that carbon dioxide is utilized even in those fermentations where there is no net uptake. This interpretation is further supported by studies on the influence of sodium fluoride on carbon dioxide exchange. Suitable concentrations of sodium fluoride

[7] *Ann. Rev. Biochem.*, **6**, 608 (1937).

inhibit carbon dioxide uptake without preventing glycerol fermentation. In those fermentations in which an evolution of carbon dioxide occurs one would expect, on the assumption that carbon dioxide is utilized, that sodium fluoride would increase the carbon dioxide production. This is found to occur. More direct evidence that carbon dioxide utilization is a general characteristic of propionic acid fermentations is provided by experiments with radioactive carbon (Carson, Foster, Ruben & Kamen).

The reactions involved in the utilization of carbon dioxide are still obscure though some possibilities have been suggested [Wood & Werkman (1, 2); Thimann]. Wood & Werkman conclude that carbon dioxide may react with some C_3 compound, possibly a phosphoric acid ester, to form succinic acid, the evidence being: (a) succinic acid production is greatly reduced in the absence of carbon dioxide; (b) succinic acid is produced and carbon dioxide is taken up in approximately equimolar quantities; (c) sodium fluoride inhibits carbon dioxide uptake, succinic acid formation, and phosphoglyceric acid decompositions similarly. The evidence is certainly not conclusive since in the glycerol fermentation the equivalence of carbon dioxide and succinic acid follows from purely stoichiometric considerations, i.e., succinic acid, being the main oxidized product, must correspond to the carbon dioxide reduced. Furthermore, it has been shown by the use of isotopes of carbon (Carson & Ruben; Wood, Werkman, Hemingway & Nier) that the carbon of carbon dioxide is converted not only into succinic acid but also into propionic acid. In experiments with radioactive carbon, the activity of each compound was approximately proportional to the amount formed. It is significant that in the fermentation of glycerol (which proceeds without detectable carbon dioxide evolution) carbon dioxide appears to be an intermediate.

The position of the carbon dioxide carbon in propionic and succinic acids is of special importance for the eventual elucidation of the mechanism of carbon dioxide assimilation. Wood, Werkman, Hemingway & Nier have shown substantially all the carbon dioxide carbon in succinic acid to be in the carboxyl groups. This led to the suggestion that carbon dioxide may react with pyruvic acid to give oxalacetic acid which is then reduced to succinic acid. Carson, Foster, Ruben & Kamen obtained evidence that seemed to indicate the fixed carbon in propionic acid occupies all possible positions. However, a reinvestigation of the problem (unpublished data) has definitely shown that in propionic acid also carbon dioxide enters only into the carboxyl

group. In view of this result the formation of propionate via succinate is possible. An interconversion of these two acids in the presence of a fermentable substrate has actually been observed, but the rate was too low to account for the total propionate formed. This and other observations suggest that succinate and propionate originate independently.

The action of sodium fluoride on the propionic acid fermentation of glucose has been further studied by Wiggert & Werkman (2). Since the bacteria will grow on glucose in the presence of sufficient sodium fluoride to completely inhibit the breakdown of phosphoglyceric acid it was previously concluded[8] that at least a part of the normal sugar fermentation does not involve phosphorylation. It is now shown that the ability to ferment sugar in the presence of sodium fluoride can be acquired by growth in media containing sodium fluoride, while the ability to ferment phosphoglyceric acid is simultaneously lost. In addition to the sodium fluoride–sensitive mechanism of succinic acid formation there appears to be a sodium fluoride–insensitive mechanism not involving carbon dioxide [Wood & Werkman (3)].

Chaix & Fromageot (2) have shown that $0.02 M$ NaF inhibits the anaerobic decomposition of glucose and lactate but not of pyruvate. The fact that the aerobic decomposition of these compounds is not influenced by the same concentrations of sodium fluoride nor by $0.0004 M$ iodoacetate has led to the conclusion that oxygen enters into the metabolism before the stage of phosphoglyceric acid. It has also been found [Chaix & Fromageot (1); Chaix] that the normal Pasteur effect in propionic acid bacteria is modified by the presence of cysteine or hydrogen sulfide so that respiration plus aerobic glycolysis is actually greater than anaerobic glycolysis (negative Pasteur effect). This is interpreted to mean that oxygen acts upon the glycolytic system in two ways: (a) by inhibiting the conversion of C_6 to C_3 compounds, and (b) by oxidizing C_3 compounds. Only (a) is influenced by sulfhydryl compounds.

The paper of Tasman & Brandwijk on the metabolism of the diphtheria bacillus may be mentioned here since it shows that the organism carries out a modified propionic acid fermentation, the products being propionic, succinic, lactic, acetic, and formic acids, ethyl alcohol, and carbon dioxide. The acids formed by the Tomcsik strain were mainly

[8] *Ann. Rev. Biochem.*, **7**, 501 (1938).

propionic and acetic, while the Bandoeng strain formed mainly acetic, lactic, and succinic. Propionic and acetic acids were also obtained by Prévot & Kirchheiner and Prévot & Veillon (3) from glucose bouillon media fermented by *Fusiformis biacutus* and *Ramibacterium ramosoides*. Since the latter organism produced considerable quantities of ammonia there is clearly no certainty that the volatile acids were derived entirely from glucose. *R. ramosum* produces formic and acetic acids but no propionic acid from sugar [Prévot & Veillon (1)].

Fermentations by the colon group.—Endo has shown that the early stages of sugar breakdown in *E. coli* are very similar to those of yeast. Experiments were carried out with acetone preparations of cells that produced acid and carbon dioxide from glucose and to a lesser extent from pyruvic acid. Glucose, hexosediphosphate, and acetaldehyde, in the absence of sodium fluoride, were converted mainly to pyruvic acid and ethanol while with sodium fluoride, phosphoglyceric acid accumulated. A point of dissimilarity to yeast was the failure of glycerophosphate to accumulate in the dissimilation of hexosediphosphate. Direct evidence that phosphorylation reactions occur also in living cells of *A. aerogenes* is provided by the observation of Wiggert & Werkman (1) that in glucose fermentations orthophosphate decreases while phosphate esters increase. The results of Stone, Mickelson & Werkman on the effect of sodium fluoride on the fermentation of glucose, phosphoglycerate, and hexosediphosphate are difficult to interpret but suggest that some system other than that of Embden and Meyerhof may be involved.

The failure of certain mutant strains of *E. coli* to ferment lactose is not due to the absence of lactase, since cells treated with antiseptics or dried show lactase activity (Deere). The greater impermeability of the variants may account for their inability to attack lactose.

Mickelson & Werkman (1, 2) find that pH has a striking effect on the fermentation products of glucose formed by *A. indologenes*. Above about pH 6.3 acetate and formate accumulate while the formation of hydrogen, carbon dioxide, and 2,3-butylene glycol is greatly decreased; below pH 6.3 the reverse is true. In acid glucose fermentations added acetate disappears with a corresponding production of 2,3-butylene glycol, suggesting a conversion of the former into the latter. Further experiments, however, have not confirmed the view that acetic acid is a necessary intermediate in 2,3-butylene glycol formation. The significant fact is that added propionic acid also increases the yield of 2,3-butylene glycol although it is itself reduced quanti-

tatively to propyl alcohol. The possibility that acetic and propionic acids function only as hydrogen acceptors is eliminated by showing that added fumarate does not influence the yield of glycol although it is reduced to succinate. Clear evidence that acetylmethylcarbinol is not formed by a simple enzymatic condensation of acetaldehyde, as is generally believed, is furnished by Silverman & Werkman (4). They show that cell-free enzyme preparations of *A. aerogenes* have no action on added acetaldehyde but convert pyruvate approximately as follows:

$$2\ CH_3 \cdot CO \cdot COOH \rightarrow 2\ CO_2 + CH_3 \cdot CO \cdot CHOH \cdot CH_3.$$
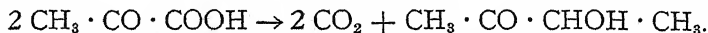
Kluyver & Molt have confirmed the earlier observation of Reynolds & Werkman[9] that *E. coli* is able to form small amounts of acetylmethylcarbinol. The quantity, however, is so small as to give a negative result with even the highly sensitive Barritt modification of the Voges-Proskauer reaction. Strong aeration of *A. indologenes* cultures increases acetylmethylcarbinol at the expense of 2,3-butylene glycol without reducing the sum of the two [Mickelson & Werkman (3)]. Added acetate is reduced in spite of the aeration.

Elsden has obtained results indicating carbon dioxide to be involved in the formation of succinate. He shows that suspensions of *E. coli,* acting upon glucose, galactose, or pyruvate, produce much more succinate at high than at low carbon dioxide tensions. Conclusive evidence that carbon dioxide can contribute to succinate formation is provided by experiments of Wood, Werkman, Hemingway & Nier using C_{13} as a tracer for carbon dioxide carbon in fermentations of galactose and pyruvate. C_{13} was present in succinate and formate but not in acetate or ethanol. Apparently all the carbon dioxide carbon in succinate is in the carboxyl groups. As in the propionic acid fermentation, the detailed mechanism of succinate formation by *E. coli* remains obscure.

Ordal & Halvorson, and Ordal & Tsuchiya have provided further support for the now widely accepted belief that *E. coli* forms hydrogen only from formate by studies of the behavior of normal and nonaerogenic variants toward glucose and formate. Failure to produce hydrogen from glucose is always associated with inability to form or to decompose formate. The possibility that hydrogen is formed by the combined action of formic dehydrogenase and hydrogenase is revived by the demonstration that *A. aerogenes* possesses both enzymes.

[9] *Ann. Rev. Biochem.,* **7,** 502 (1938).

In glycerol fermentations by *B. freundii* the yields of trimethylene glycol vary from 36.0 to 60.4 per cent, though the cause of these variations is not clear [Mickelson & Werkman (5)]. It is somewhat surprising that added fumarate is reduced to succinate without lowering the yield of trimethylene glycol, since fumarate might be expected to compete with glycerol for the available hydrogen. Other products of glycerol fermentation are hydrogen, carbon dioxide, formate, acetate, lactate, succinate, and ethanol, as was previously shown by Braak. Acrolein, pyruvate, and acetaldehyde are suggested as possible intermediates in this fermentation since they can be isolated by dimedon or sulfite fixation (Reynolds, Hoehn & Werkman). However, the intermediate role of acrolein is open to considerable doubt, since added acrolein is very toxic and appears not to be metabolized. Of taxonomic interest is the fermentation of glycerol in a mineral medium by *A. aerogenes* with the formation of 31 to 45 per cent trimethylene glycol and about 10 per cent butylene glycol [Mickelson & Werkman (4)]. Previously it was thought that only colon group "intermediates" could form trimethylene glycol.

The fermentation of citrate in a mineral medium by *Aerobacter indologenes, A. aerogenes,* or *Ps. pyocyaneus* gives rise chiefly to acetic, formic, and succinic acids and carbon dioxide [Deffner; Deffner & Franke; Brewer & Werkman (1)]. Although the mechanism of this fermentation is still largely hypothetical, the products can be most easily accounted for by supposing that citrate is first split into acetate and oxalacetate. The latter compound is shown to yield the same products as citrate, less one mol of acetic acid. Mechanisms involving the primary formation of either aconitic acid or acetonedicarboxylic acid from citrate appear to be excluded by the inability of the bacteria to ferment these compounds.

Other carbohydrate fermentations.—Friedemann (1, 2, 3, 4) finds that a number of pathogenic bacteria carry out a lactic acid fermentation of glucose. With streptococci, pneumococci, and staphylococci, in particular, lactic acid accounts for 70 to 90 per cent of the glucose fermented. Smaller quantities of formate, acetate, and ethanol are formed in a molecular ratio of approximately 2 to 1 to 1. The yield of lactic and volatile acids in rapidly growing cultures of pneumococci is largely unaffected by moderate aeration.

Precise chemical information is at last accumulating on sugar fermentations by so-called "putrefactive" anaerobes. Clifton (2) finds that ethanol and carbon dioxide account for 50 to 65 per cent of the

glucose fermented by *Cl. botulinum* Types A and B. Small amounts of acetic and lactic acids and hydrogen are also formed; 25 per cent of the substrate carbon still is unaccounted for. Pyruvate [Clifton (1)] is fermented by the same organisms approximately according to the equation:

$$2\,CH_3 \cdot CO \cdot COOH + H_2O \rightarrow 2\,CO_2 + C_2H_5OH + CH_3 \cdot COOH$$
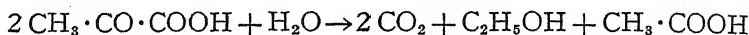
though the yields of the oxidized products, acetic acid, and carbon dioxide, are somewhat too high and no corresponding reduction products were found. *Cl. tetanomorphum* (Woods & Clifton) decomposes pyruvate in a different manner, the approximate equation being:

$$3\,CH_3 \cdot CO \cdot COOH + H_2O \rightarrow$$
$$CH_3 \cdot COOH + CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH + 3\,CO_2 + H_2.$$
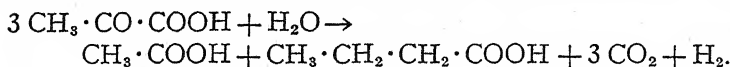
Small amounts of lactate are also produced.

Plectridium pseudotetanicum [Prévot & Veillon (2)] forms considerable acetate and a little ethanol and lactate from glucose. *Pl. tetani* (Boorsma, Prévot & Veillon), a species usually considered unable to ferment sugars, is claimed to attack glucose in the presence of bouillon with the formation of carbon dioxide, hydrogen, acetic, propionic, and butyric acids, and methyl, ethyl, propyl, and butyl alcohols. Since the carbon in these products is considerably greater than in the glucose fermented, some of them must have been derived from other constituents of the medium. However, the assumption of Boorsma *et al.* that only propionic acid and propanol originated in this way appears unsupported by evidence.

Prévot & Pochon claim to have isolated a new cellulose-fermenting *Plectridium* which requires peptone or other complex nitrogenous substances. The products of cellulose decomposition are mainly acetic and butyric acids with a little carbon dioxide, hydrogen, and ethanol. The reported yield of volatile acids (88 per cent of the cellulose carbon) is surprisingly high in view of current theories of the mechanism of carbohydrate breakdown, but is not inconsistent with earlier results of Omelianski and of Viljoen, Fred & Peterson.

Anaerobic breakdown of nitrogenous compounds.—Present information on the anaerobic breakdown of nitrogenous compounds clearly indicates that two types of oxidation-reduction reactions may be involved: (*a*) reactions between unlike substrate molecules and (*b*) reactions between like substrate molecules or between a substrate and its decomposition products. Obviously there is no fundamental chemi-

cal difference between these two types of catabolic processes. Both may be carried out by one organism.

Both cell suspensions and growing cultures of *Cl. tetanomorphum* and *Cl. cochlearium* have been found to ferment glutamic acid with production of butyric and acetic acids, carbon dioxide, hydrogen, and ammonia [Woods & Clifton; Barker (1, 2)]. The relative quantities of these products vary with the experimental conditions. Raising the pH increases hydrogen and decreases butyric acid production. This effect of pH is just opposite to that observed with *A. indologenes* by Mickelson & Werkman (1). It is significant that some substrates decomposed by cell suspensions (aspartic and fumaric acids) appear to be unable to support the growth of *Cl. tetanomorphum*.

Clifton (1, 2) finds that *Cl. botulinum* Types A and B, like *Cl. sporogenes,* is able to carry out oxidation-reduction reactions between pairs of different amino acids. Phenylalanine, leucine, lysine, glutamic acid, and alanine are used as hydrogen donors, the latter being oxidized to acetic acid. Glycine and proline, acting as hydrogen acceptors, are reduced to acetic acid and δ-amino valeric acid, respectively. Leucine or serine alone is slowly fermented [Clifton (3)]. The products of serine decomposition are approximately expressed by the equation:

$$2\ CH_2OH \cdot CHNH_2 \cdot COOH + H_2O \rightarrow$$
$$2\ NH_3 + 2\ CO_2 + C_2H_5OH + CH_3 \cdot COOH.$$
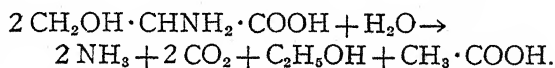
The formation of butyric acid by *Cl. botulinum* growing in complex media has not yet been explained.

A little known and highly specialized organism, *Cl. acidi-urici*, is capable of rapidly fermenting uric acid, xanthine, hypoxanthine, and guanine under strictly anaerobic conditions with formation of ammonia, carbon dioxide, and acetic acid (Beck; Barker, Ruben & Beck). Since the yield of acetic acid from hypoxanthine (1.10 to 1.25 mols per mol) is higher than can be accounted for by a direct decomposition, the possibility that acetic acid is synthesized from carbon dioxide was tested by the use of radioactive carbon. Carbon dioxide carbon was found in both carboxyl and methyl groups indicating a total synthesis of acetic acid. It is suggested that these fermentations may be "complete oxidations of purines with carbon dioxide acting as the ultimate oxidizing agent." The generalized equation for such a metabolic process would be:

$$4\ H_2A + 2\ CO_2 \rightarrow 4\ A + CH_3 \cdot COOH + 2\ H_2O.$$
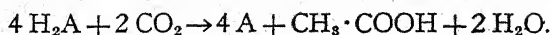
The number of substances known to be oxidized or reduced by *Cl. sporogenes*[10] has been greatly increased (Kocholaty & Hoogerheide; Hoogerheide & Kocholaty). More than forty substances can act as hydrogen donors. Generally most active in this respect are amino acids, though under certain conditions ethanol is the best hydrogen donor. It is of particular interest that the activation of various compounds by cell suspensions is greatly influenced by environmental factors and previous cultural conditions. The ability of *Cl. sporogenes* to use gaseous hydrogen as a reducing agent was used in the detection of hydrogen acceptors. Besides the amino acids previously recognized as hydrogen acceptors many other nitrogenous and nonnitrogenous compounds are reduced more slowly. Proline, hydroxyproline, glycine, ornithine, and tryptophane are apparently reduced by hydrogen to δ-amino valeric, γ-hydroxy-δ-amino valeric, acetic, δ-amino valeric, and indole propionic acids, respectively. Serine, arginine, cysteine, cystine, methionine, tryptophane, pyruvate, and glucose are fermented in the absence of other hydrogen donors or acceptors. Hydrogen is evolved only from glucose; this is in contrast to results with *Cl. tetanomorphum*.

Methane fermentation.—The study of the biochemical activities of methane-producing bacteria has been greatly facilitated by the isolation of pure cultures of two species, *Methanosarcina methanica* and *Methanobacterium omelianskii* [Kluyver & Schnellen; Barker (3)]. The latter organism has been shown to oxidize simple primary and secondary alcohols only as far as the corresponding fatty acids and ketones, carbon dioxide being simultaneously reduced to methane [Barker (4); see also Kluyver]. Neither formate, methanol, nitrate, sulfate, nor oxygen can replace carbon dioxide as an oxidizing agent, though formate can under certain circumstances be decomposed by this organism. The evidence indicates that formate and methanol are not intermediates in the reduction of carbon dioxide to methane. During the growth of *Mb. omelianskii* about 6 per cent of the substrate (ethanol plus carbon dioxide) carbon is converted into cell material. By means of carbon balance experiments and by the use of radioactive carbon (as carbon dioxide) it is shown that most of the cell carbon is derived from ethanol, only about 10 per cent coming from carbon dioxide. The generalization of van Niel that methane is formed by reduction of carbon dioxide is shown for the first time to apply to a

[10] *Ann. Rev. Biochem.,* **7,** 507 (1938).

fermentation involving no net uptake of carbon dioxide; when *Ms. methanica* decomposes methanol in the presence of radioactive carbon dioxide, the resulting methane is radioactive (Barker, Ruben & Kamen).

BACTERIAL PHOTOSYNTHESIS

According to van Niel's view of the metabolism of the nonsulfur purple bacteria (Athiorhodaceae), the required organic compounds serve primarily as hydrogen donors for the photochemical reduction of carbon dioxide; but heretofore it has not been possible to obtain direct evidence for this interpretation because of the complexity of the reactions undergone by all known organic substrates. Foster has now supplied the missing evidence by isolating and studying an organism whose photosynthetic metabolism involves a simple dehydrogenation of isopropanol according to the equation:

$$2\,CH_3 \cdot CHOH \cdot CH_3 + CO_2 \rightarrow 2\,CH_3 \cdot CO \cdot CH_3 + (CH_2O) + H_2O.$$
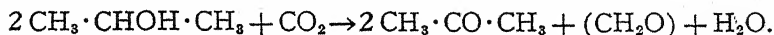
Since the acetone recovered is closely equivalent to the alcohol oxidized, the cell material [represented by (CH_2O)] clearly must have been derived mainly from carbon dioxide.

The absorption spectra of photosynthetic bacteria have received considerable attention, particularly with regard to the relation between the bacteriochlorophyll spectra of living cells and of various cell-free extracts. It has long been known that when chlorophyll is extracted from green plants with organic solvents, the absorption maximum in the red wave length region shifts to shorter wave lengths as compared to its position in the plastids. An even greater shift in this direction occurs as a result of the extraction of the green pigment of purple (Thiorhodaceae and Athiorhodaceae) and green bacteria with organic solvents (Vermeulen, Wassink & Reman; Katz & Wassink). The infrared absorption spectra of alcoholic extracts of various groups of photosynthetic bacteria are essentially identical in shape and position, whereas the spectra of intact cells of Thio- and Athiorhodaceae in general differ significantly. This and other observations suggest that "in purple bacteria the same bacteriochlorophyll is, in the intact cell, bound to different proteins, thus building up different photoactive complexes characterized by somewhat different infrared absorption spectra" (Wassink, Katz & Dorrestein). Colloidal solutions of a protein-bacteriochlorophyll complex have been prepared which possess absorption spectra very similar to living cells [Katz & Was-

sink; French (2, 3)]. Such solutions do not carry on photosynthesis but can act as a photocatalyst for the oxidation of ascorbic acid with visible and infrared radiation.

One of the fundamental differences between bacterial and green plant photosynthesis is the absence of oxygen production in the former. Nakamura's (1) claim to have demonstrated the formation of oxygen by *Rhodobacillus* has been shown by van Niel to be fallacious. The ability of *Chromatium* to take up and produce hydrogen in the presence of various organic and inorganic compounds is of interest [Nakamura (2, 3)], but the view that sulfur accumulation in the Thiorhodaceae is primarily due to the absence of a sulfur-reducing enzyme appears to be entirely unsound. The claim that sulfate is rapidly reduced to hydrogen sulfide by hydrogen in the dark is not proved and also is inconsistent with careful and extensive experiments by van Niel.[11]

For the photoreduction of one mol of carbon dioxide by *Streptococcus varians,* 2.6 instead of 2.0 mols of hydrogen are used (Wessler & French). This raises the calculated number of quanta required per molecule of carbon dioxide assimilated from 4 to 5 [French (1)].

[11] *Ann. Rev. Biochem.,* **6,** 606 (1937).

LITERATURE CITED

ADLER, E., HELLSTRÖM, V., GÜNTHER, G., AND EULER, H. v., *Z. physiol. Chem.*, **255**, 14 (1938)

ALLISON, F. E., AND MINOR, F. W., (1), *Soil Sci.*, **46**, 473 (1938)

ALLISON, F. E., AND MINOR, F. W., (2), *J. Bact.*, **39**, 373 (1940)

ANONYMOUS, *Nature*, **146**, 139 (1940)

BAKER, J. W., AND HAPPOLD, F. C., *Biochem. J.*, **34**, 657 (1940)

BARKER, H. A., (1), *Enzymologia*, **2**, 175 (1937)

BARKER, H. A., (2), *Arch. Mikrobiol.*, **10**, 376 (1939)

BARKER, H. A., (3), *Antonie van Leeuwenhoek*, **6**, 201 (1940)

BARKER, H. A., (4), *J. Biol. Chem.*, **137**, 153 (1941)

BARKER, H. A., RUBEN, S., AND BECK, J. V., *Proc. Natl. Acad. Sci. U.S.*, **26**, 477 (1940)

BARKER, H. A., RUBEN, S., AND KAMEN, M. D., *Proc. Natl. Acad. Sci. U.S.*, **26**, 426 (1940)

BARRITT, M. M., *J. Path. Bact.*, **44**, 679 (1937)

BARRON, E. S. G., AND JACOBS, H. R., *J. Bact.*, **36**, 433 (1938)

BARRON, E. S. G., AND LYMAN, C. M., *J. Biol. Chem.*, **127**, 143 (1939)

BECK, J. V., *Thesis* (University of California, 1940)

BERNHAUER, K., *Ergeb. Enzymforsch.*, **7**, 246 (1938)

BERNHAUER, K., AND KNOBLOCH, H., *Biochem. Z.*, **303**, 308 (1940)

BJÄLFVE, G., NILSSON, R., AND BURSTRÖM, D., *Ann. Landw. Hochschule Schwedens*, **7**, 291 (1939)

BOORSMA, H. J., PRÉVOT, A. R., AND VEILLON, R., *Compt. rend. soc. biol.*, **131**, 1137 (1939)

BOOTH, V. H., AND GREEN, D. E., *Biochem. J.*, **32**, 855 (1938)

BRAAK, H. R., *Dissertation* (Groningen, 1928)

BREWER, C. R., AND WERKMAN, C. H., (1), *Enzymologia*, **6**, 273 (1939)

BREWER, C. R., AND WERKMAN, C. H., (2), *Proc. Soc. Exptl. Biol. Med.*, **41**, 185 (1939)

BROWN, R. W., STAHLY, G. L., AND WERKMAN, C. H., *Iowa State Coll. J. Sci.*, **12**, 245 (1938)

BROWN, R. W., WOOD, H. G., AND WERKMAN, C. H., *J. Bact.*, **38**, 631 (1939)

BURROWS, W., (1), *J. Infectious Diseases*, **64**, 145 (1939)

BURROWS, W., (2), *J. Infectious Diseases*, **65**, 134 (1939)

BUTLIN, K. R., (1), *Biochem. J.*, **32**, 508 (1938)

BUTLIN, K. R., (2), *Biochem. J.*, **32**, 1185 (1938)

BUTLIN, K. R., (3), *J. Soc. Chem. Ind.*, **57**, 463 (1938)

BUTLIN, K. R., AND WINCE, W. H. D., (1), *J. Soc. Chem. Ind.*, **58**, 363 (1939)

BUTLIN, K. R., AND WINCE, W. H. D., (2), *J. Soc. Chem. Ind.*, **58**, 365 (1939)

CARSON, S. F., FOSTER, J. W., RUBEN, S., AND KAMEN, M. D., *Science*, **92**, 433 (1940)

CARSON, S. F., AND RUBEN, S., *Proc. Natl. Acad. Sci. U.S.*, **26**, 422 (1940)

CHAIX, P., *Thesis* (Bosc Frères M. and L. Riou, Lyon, 1940)

CHAIX, P., AND FROMAGEOT, C., (1), *Enzymologia*, **6**, 33 (1939)

CHAIX, P., AND FROMAGEOT, C., (2), *Enzymologia*, **7**, 353 (1939)

CLAREN, O. B., *Ann.*, **535**, 122 (1938)

CLIFTON, C. E., (1), *Proc. Soc. Exptl. Biol. Med.,* **43**, 585 (1940)

CLIFTON, C. E., (2), *J. Bact.,* **39**, 485 (1940)

CLIFTON, C. E., (3), *Proc. Soc. Exptl. Biol. Med.,* **43**, 588 (1940)

CLIFTON, C. E., AND LOGAN, W. A., *J. Bact.,* **37**, 523 (1939)

DEERE, C. J., *J. Bact.,* **37**, 473 (1939)

DEFFNER, M., *Ann.,* **536**, 44 (1938)

DEFFNER, M., AND FRANKE, W., *Ann.,* **541**, 85 (1939)

DESNUELLE, P., (1), *Enzymologia,* **6**, 242 (1939)

DESNUELLE, P., (2), *Enzymologia,* **6**, 387 (1939)

DESNUELLE, P., AND FROMAGEOT, C., *Enzymologia,* **6**, 80 (1939)

DESNUELLE, P., WOOKEY, E., AND FROMAGEOT, C., *Enzymologia,* **8**, 225 (1940)

DORFMAN, A., KOSER, S. A., HORWITT, M. K., BERKMAN, S., AND SAUNDERS, F., *Proc. Soc. Exptl. Biol. Med.,* **43**, 434 (1940)

DORFMAN, A., KOSER, S. A., REAMES, H. R., SWINGLE, K. F., AND SAUNDERS, F., *J. Infectious Diseases,* **65**, 163 (1939)

DORFMAN, A., KOSER, S. A., AND SAUNDERS, F., (1), *J. Am. Chem. Soc.,* **60**, 2004 (1938)

DORFMAN, A., KOSER, S. A., AND SAUNDERS, F., (2), *Science,* **90**, 544 (1939)

DUBOS, R. J., AND MILLER, B. F., *Proc. Soc. Exptl. Biol. Med.,* **39**, 65 (1938)

EAGLES, B. A., OKULITCH, O., AND KADZIELAWA, A. S., *Can. J. Research, B,* **16**, 46 (1938)

EGAMI, M. F., *Bull. soc. chim. biol.,* **20**, 301 (1938)

EGGERTH, A. N., *J. Bact.,* **37**, 205 (1939)

ELBERG, S. S., AND MEYER, K. F., *J. Bact.,* **37**, 429 (1939)

ELSDEN, S. R., *Biochem. J.,* **32**, 187 (1938)

ENDO, S., *Biochem. Z.,* **296**, 56 (1938)

EVANS, W. C., HANDLEY, W. R. C., AND HAPPOLD, F. C., *Brit. J. Exptl. Path.,* **20**, 396 (1939)

EVANS, W. C., HAPPOLD, F. C., AND HANDLEY, W. R. C., *Brit. J. Exptl. Path.,* **20**, 41 (1939)

FILDES, P., (1), *Brit. J. Exptl. Path.,* **19**, 239 (1938)

FILDES, P., (2), *Biochem. J.,* **32**, 1600 (1938)

FILDES, P., AND GLADSTONE, G. P., *Brit. J. Exptl. Path.,* **20**, 334 (1939)

FOSTER, J. W., *J. Gen. Physiol.,* **24**, 123 (1940)

FRENCH, C. S., (1), *J. Gen. Physiol.,* **20**, 711 (1937)

FRENCH, C. S., (2), *J. Gen. Physiol.,* **23**, 469 (1940)

FRENCH, C. S., (3), *J. Gen. Physiol.,* **23**, 483 (1940)

FRIEDEMANN, T. E., (1), *J. Bact.,* **35**, 527 (1938)

FRIEDEMANN, T. E., (2), *J. Biol. Chem.,* **130**, 61 (1939)

FRIEDEMANN, T. E., (3), *Proc. Soc. Exptl. Biol. Med.,* **40**, 505 (1939)

FRIEDEMANN, T. E., (4), *J. Biol. Chem.,* **130**, 757 (1939)

FROMAGEOT, C., AND BOST, G., *Enzymologia,* **2**, 225 (1938)

FROMAGEOT, C., AND SAFAVI, R., *Enzymologia,* **6**, 57 (1939)

FULMER, E. I., DUNNING, J. W., AND UNDERKOFLER, L. A., *Iowa State Coll. J. Sci.,* **13**, 279 (1939)

GALE, E. F., (1), *Biochem. J.,* **32**, 1583 (1938)

GALE, E. F., (2), *Biochem. J.,* **33**, 1012 (1939)

GALE, E. F., (3), *Biochem. J.,* **34**, 392 (1940)

GALE, E. F., (4), *Biochem. J.*, **34**, 846 (1940)

GALE, E. F., (5), *Biochem. J.*, **34**, 853 (1940)

GALE, E. F., AND STEPHENSON, M., (1), *Biochem. J.*, **32**, 392 (1938)

GALE, E. F., AND STEPHENSON, M., (2), *Biochem. J.*, **33**, 1245 (1939)

GLADSTONE, G. P., *Brit. J. Exptl. Path.*, **20**, 189 (1939)

HANN, R. M., TILDEN, E. B., AND HUDSON, C. S., *J. Am. Chem. Soc.*, **60**, 1201 (1938)

HANSEN, P. A., *Zentr. Bakt. Parasitenk.*, II, **98**, 289 (1938)

HES, J. W., *Nature*, **141**, 647 (1938)

HILLS, G. M., (1), *Biochem. J.*, **32**, 383 (1938)

HILLS, G. M., (2), *Biochem. J.*, **34**, 1057 (1940)

HOOGERHEIDE, J. C., AND KOCHOLATY, W., *Biochem. J.*, **32**, 949 (1938)

HOOVER, S. R., AND ALLISON, F. E., *J. Biol. Chem.*, **134**, 181 (1940)

HUTCHINGS, B. L., AND WOOLLEY, D. W., *Science*, **90**, 41 (1939)

HUTNER, S. H., *J. Bact.*, **35**, 429 (1938)

KATAGIRI, H., AND KITAHARA, K., *Biochem. J.*, **32**, 1654 (1938)

KATAGIRI, H., AND MURAKAMI, S., *Biochem. J.*, **33**, 1257 (1939)

KATZ, E., AND WASSINK, E. C., *Enzymologia*, **7**, 97 (1939)

KEMPNER, W., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 269 (1939)

KLEIN, J. R., *J. Biol. Chem.*, **134**, 43 (1940)

KLIGLER, I. J., AND GROSOWITZ, N., *J. Bact.*, **38**, 309 (1939)

KLUYVER, A. J., *Suomen Kemistilehti*, **5**, 81 (1939)

KLUYVER, A. J., AND BOEZAARDT, A. G. J., (1), *Rec. trav. chim.*, **57**, 609 (1938)

KLUYVER, A. J., AND BOEZAARDT, A. G. J., (2), *Rec. trav. chim.*, **58**, 956 (1939)

KLUYVER, A. J., AND MOLT, E. L., *Proc. Acad. Sci. Amsterdam*, **42**, 118 (1939)

KLUYVER, A. J., AND SCHNELLEN, C. (Private communication)

KNIGHT, B. C. J. G., AND McILWAIN, H., *Biochem. J.*, **32**, 1241 (1938)

KOCHOLATY, W., AND HOOGERHEIDE, J. C., *Biochem J.*, **32**, 437 (1938)

KOCHOLATY, W., SMITH, L. D., AND WEIL, L., *Biochem. J.*, **32**, 1691 (1938)

KÖGL, F., AND VAN WAGTENDONK, W. J., *Rec. trav. chim.*, **57**, 747 (1938)

KOSER, S. A., AND SAUNDERS, F., *Bact. Rev.*, **2**, 99 (1938)

KRAUSKOPF, E. J., SNELL, E. E., AND McCOY, E., *Enzymologia*, **7**, 327 (1939)

KREBS, H. A., AND EGGLESTON, L. V., *Enzymologia*, **7**, 310 (1939)

LANDY, M., (1), *Proc. Soc. Exptl. Biol. Med.*, **38**, 504 (1938)

LANDY, M., (2), *Nature*, **144**, 512 (1939)

LIEBERT, F., *Proc. Acad. Sci. Amsterdam*, **12**, 54 (1910)

LIPMANN, F., (1), *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 248 (1939)

LIPMANN, F., (2), *J. Biol. Chem.*, **134**, 463 (1940)

LWOFF, A., *Ann. inst. Pasteur*, **61**, 580 (1938)

LWOFF, A., AND QUERIDO, A., (1), *Compt. rend. soc. biol.*, **129**, 1039 (1938)

LWOFF, A., AND QUERIDO, A., (2), *Compt. rend. soc. biol.*, **130**, 1569 (1939)

McDANIEL, L. E., WOOLLEY, D. W., AND PETERSON, W. H., *J. Bact.*, **37**, 259 (1939)

McILWAIN, H., (1), *Brit. J. Exptl. Path.*, **20**, 330 (1939)

McILWAIN, H., (2), *Biochem. J.*, **33**, 1942 (1939)

McILWAIN, H., (3), *Brit. J. Exptl. Path.*, **21**, 25 (1940)

McILWAIN, H., FILDES, P., GLADSTONE, G. P., AND KNIGHT, B. C. J. G., *Biochem. J.*, **33**, 223 (1939)

MICKELSON, M., AND WERKMAN, C. H., (1), *J. Bact.*, **36**, 67 (1938)

MICKELSON, M. N., AND WERKMAN, C. H., (2), *J. Bact.*, **37**, 619 (1939)

MICKELSON, M. N., AND WERKMAN, C. H., (3), *Iowa State Coll. J. Sci.*, **13**, 157 (1939)

MICKELSON, M. N., AND WERKMAN, C. H., (4), *Enzymologia*, **8**, 252 (1940)

MICKELSON, M. N., AND WERKMAN, C. H., (5), *J. Bact.*, **39**, 709 (1940)

MILLER, B. F., ALLINSON, M. J. C., AND BAKER, Z., *J. Biol. Chem.*, **130**, 383 (1939)

MITCHELL, H. K., SNELL, E. E., AND WILLIAMS, R. J., *J. Am. Chem. Soc.*, **62**, 1791 (1940)

MÖLLER, E. F., (1), *Z. physiol. Chem.*, **254**, 285 (1938)

MÖLLER, E. F., (2), *Z. physiol. Chem.*, **260**, 246 (1939)

MUELLER, J. H., (1), *J. Bact.*, **36**, 499 (1938)

MUELLER, J. H., (2), *J. Biol. Chem.*, **123**, 421 (1938)

MUELLER, J. H., AND KLOTZ, A. W., *J. Am. Chem. Soc.*, **60**, 3086 (1938)

NAKAMURA, H., (1), *Acta Phytochim. (Japan)*, **9**, 189 (1937)

NAKAMURA, H., (2), *Acta Phytochim. (Japan)*, **10**, 259 (1938)

NAKAMURA, H., (3), *Acta Phytochim. (Japan)*, **11**, 109 (1939)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (1), *Ann. Landw. Hochschule Schwedens*, **5**, 291 (1938)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (2), *Ann. Landw. Hochschule Schwedens*, **7**, 301 (1939)

OMELIANSKI, V., *Zentr. Bakt. Parasitenk.*, *II*, **8**, 353 (1902)

ORDAL, E. J., AND HALVORSON, H. O., *J. Bact.*, **38**, 199 (1939)

ORDAL, E. J., AND TSUCHIYA, H. M., *Rept. Proc. 3rd Intern. Congr. Microbiol.*, 217 (1939)

ORLA-JENSEN, S., AND SNOG-KJAER, A., *Rept. Proc. 3rd Intern. Congr. Microbiol.*, 501 (1939)

PAN, S. C., PETERSON, W. H., AND JOHNSON, M. J., *Ind. Eng. Chem.*, *Ind. Ed.*, **32**, 709 (1940)

PAPPENHEIMER, JR., A. M., AND HOTTLE, G. A., *Proc. Soc. Exptl. Biol. Med.*, **44**, 645 (1940)

PELCZAR, JR., M. J., AND PORTER, J. R., (1), *J. Bact.*, **39**, 429 (1940)

PELCZAR, JR., M. J., AND PORTER, J. R., (2), *Proc. Soc. Exptl. Biol. Med.*, **43**, 151 (1940)

PENNINGTON, D., SNELL, E. E., AND WILLIAMS, R. J., *J. Biol. Chem.*, **135**, 213 (1940)

PHELPS, A. S., JOHNSON, M. J., AND PETERSON, W. H., (1), *Biochem. J.*, **33**, 726 (1939)

PHELPS, A. S., JOHNSON, M. J., AND PETERSON, W. H., (2), *Biochem. J.*, **33**, 1606 (1939)

PORTER, J. R., AND PELCZAR, JR., M. J., *Science*, **91**, 576 (1940)

PRATT, E. F., AND WILLIAMS, R. J., *J. Gen. Physiol.*, **22**, 637 (1939)

PRÉVOT, A. R., AND KIRCHHEINER, E., *Compt. rend. soc. biol.*, **128**, 963 (1938)

PRÉVOT, A. R., AND POCHON, J., *Compt. rend. soc. biol.*, **130**, 966 (1939)

PRÉVOT, A. R., AND VEILLON, R., (1), *Compt. rend. soc. biol.*, **128**, 451 (1938)

PRÉVOT, A. R., AND VEILLON, R., (2), *Compt. rend. soc. biol.*, **129**, 931 (1938)

PRÉVOT, A. R., AND VEILLON, R., (3), *Compt. rend. soc. biol.*, **132**, 239 (1939)

RANE, L., AND SUBBAROW, Y., (1), *Proc. Soc. Exptl. Biol. Med.,* **38**, 837 (1938)

RANE, L., AND SUBBAROW, Y., (2), *J. Biol. Chem.,* **134**, 455 (1940)

REYNOLDS, H., HOEHN, W. M., AND WERKMAN, C. H., *Iowa State Coll. J. Sci.,* **13**, 275 (1939)

RIPPEL, A., AND NABEL, K., *Arch. Mikrobiol.,* **10**, 359 (1939)

RUBEN, S., AND KAMEN, M. D., *Proc. Natl. Acad. Sci. U.S.,* **26**, 418 (1940)

SCHÜTZ, F., AND THEORELL, H., *Biochem. Z.,* **295**, 246 (1938)

SILVERMAN, M., AND WERKMAN, C. H., (1), *J. Bact.,* **38**, 25 (1939)

SILVERMAN, M., AND WERKMAN, C. H., (2), *Proc. Soc. Exptl. Biol. Med.,* **40**, 369 (1939)

SILVERMAN, M., AND WERKMAN, C. H., (3), *Iowa State Coll. J. Sci.,* **13**, 107 (1939)

SILVERMAN, M., AND WERKMAN, C. H., (4), *Proc. Soc. Exptl. Biol. Med.,* **43**, 777 (1940)

SNELL, E. E., AND PETERSON, W. H., *J. Bact.,* **39**, 273 (1940)

SNELL, E. E., AND STRONG, F. M., (1), *Ind. Eng. Chem., Anal. Ed.,* **11**, 346 (1939)

SNELL, E. E., AND STRONG, F. M., (2), *Enzymologia,* **6**, 186 (1939)

SNELL, E. E., STRONG, F. M., AND PETERSON, W. H., (1), *J. Am. Chem. Soc.,* **60**, 2825 (1938)

SNELL, E. E., STRONG, F. M., AND PETERSON, W. H., (2), *J. Bact.,* **38**, 293 (1939)

SNELL, E. E., AND WILLIAMS, R. J., *J. Am. Chem. Soc.,* **61**, 3594 (1939)

STEPHENSON, M., AND TRIM, A. R., *Biochem. J.,* **32**, 1740 (1938)

STONE, R. W., MICKELSON, M. N., AND WERKMAN, C. H., *Iowa State Coll. J. Sci.,* **14**, 253 (1940)

SUBBAROW, Y., AND RANE, L., *J. Am. Chem. Soc.,* **61**, 1616 (1939)

TARR, H. L. A., *J. Fisheries Research Board Can.,* **4**, 367 (1939)

TASMAN, A., AND BRANDWIJK, A. C., *J. Infectious Diseases,* **63**, 10 (1938)

TATUM, E. L., PETERSON, W. H., AND FRED, E. B., *J. Bact.,* **29**, 563 (1935)

THIMANN, K. V., *Science,* **88**, 506 (1938)

TILDEN, E. B., *J. Bact.,* **37**, 629 (1939)

VAN NIEL, C. B., *Rept. Proc. 3rd Intern. Congr. Microbiol.,* 247 (1939)

VERMEULEN, D., WASSINK, E. C., AND REMAN, G. H., *Enzymologia,* **4**, 254 (1937)

VILJOEN, J. A., FRED, E. B., AND PETERSON, W. H., *J. Agr. Sci.,* **16**, 1 (1926)

WARBURG, O., AND CHRISTIAN, W., *Naturwissenschaften,* **26**, 201 (1938)

WASSINK, E. C., KATZ, E., AND DORRESTEIN, R., *Enzymologia,* **7**, 113 (1939)

WEBSTER, M. D., *Proc. Soc. Exptl. Biol. Med.,* **40**, 289 (1939)

WEIZMANN, C., AND ROSENFELD, B., *Biochem. J.,* **33**, 1376 (1939)

WESSLER, S., AND FRENCH, C. S., *J. Cellular Comp. Physiol.,* **13**, 327 (1939)

WEST, P. M., AND WILSON, P. W., (1), *J. Bact.,* **37**, 161 (1939)

WEST, P. M., AND WILSON, P. W., (2), *Enzymologia,* **8**, 152 (1940)

WHISTLER, R. L., AND UNDERKOFLER, L. A., *J. Am. Chem. Soc.,* **60**, 2507 (1938)

WIELAND, H., AND PISTOR, H. T., *Ann.,* **535**, 205 (1938)

WIGGERT, W. P., AND WERKMAN, C. H., (1), *Biochem. J.,* **32**, 101 (1938)

WIGGERT, W. P., AND WERKMAN, C. H., (2), *Biochem. J.,* **33**, 1061 (1939)

WIGGERT, W. P., SILVERMAN, M., UTTER, M. F., AND WERKMAN, C. H., *Iowa State Coll. J. Sci.,* **14,** 179 (1940)

WOOD, H. G., ANDERSON, A. A., AND WERKMAN, C. H., *J. Bact.,* **36,** 201 (1938)

WOOD, H. G., AND WERKMAN, C. H., (1), *Biochem. J.,* **32,** 1262 (1938)

WOOD, H. G., AND WERKMAN, C. H., (2), *Biochem. J.,* **34,** 7 (1940)

WOOD, H. G., AND WERKMAN, C. H., (3), *Biochem. J.,* **34,** 129 (1940)

WOOD, H. G., WERKMAN, C. H., HEMINGWAY, A., AND NIER, A. O., *J. Biol. Chem.,* **135,** 789 (1940)

WOODS, D. D., AND CLIFTON, C. E., *Biochem. J.,* **32,** 345 (1938)

WOOLLEY, D. W., *J. Biol. Chem.,* **130,** 417 (1939)

WOOLLEY, D. W., AND HUTCHINGS, B. L., (1), *J. Bact.,* **38,** 285 (1939)

WOOLLEY, D. W., AND HUTCHINGS, B. L., (2), *J. Bact.,* **39,** 387 (1940)

WOOLLEY, D. W., MCDANIEL, L. E., AND PETERSON, W. H., *J. Biol. Chem.,* **131,** 381 (1939)

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BIOCHEMICAL NITROGEN FIXATION

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The present chapter on biochemical nitrogen fixation is the first to appear in the ten volumes of the *Annual Review of Biochemistry* now published. The report will therefore be obliged to cover in some measure a decade of work in this field. Attention will be focussed mainly on the last four years, with, however, no intended omission of important literature in the earlier six. Fortunately for the reviewing, the subject of biochemical nitrogen fixation, one of quite general interest for the past hundred years, has made its advances more by decades than by years, so to speak, and latterly has been investigated intensively by relatively few schools of workers, in particular—though by no means exclusively—by those schools abroad at Helsinki (Biochemical Institute), Brie-Comte-Robert (Pasteur Institute), Berlin-Dahlem (Biologische Reichsanstalt), Ultuna (Institute of Microbiology, Agricultural College of Sweden), and Harpenden (Rothamsted Experimental Station), and in this country at Madison (Department of Agricultural Bacteriology, University of Wisconsin), Washington (former Fixed Nitrogen Research Laboratory), and intermittently at some half dozen state agricultural experiment stations. This circumstance of intensive localization in the study of the biochemical aspects of biological nitrogen fixation facilitates current crystallization of the main developments and controversies in the field, and evaluation of the surprisingly numerous revisions of both interpretation and even observation that have transpired. With a view to ironing out many of the existing differences of viewpoint and conclusion, the reviewing has been undertaken by erstwhile representatives of both the Washington and Madison schools. Superseding corrections of pre-

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viously accepted findings or tenets will be made in important instances, often with categorical brevity.

The phases of biochemical nitrogen fixation to be considered here, as being of greatest interest at present, and as involving either the most controversy, significant progress, or approximate resolution, are the effects of different gases (nitrogen, oxygen, hydrogen, carbon monoxide, carbon dioxide, helium, argon) ; the isotopic approach (N^{13}, N^{15}) ; excretion of fixed nitrogen compounds ; fixation or anabolic nitrogenous intermediates ; inorganic nutrition possibly specific for fixation (iron, molybdenum-vanadium, calcium-strontium, pH) ; nitrogen nutrition ; and growth factors. Limitations of space compel restriction of this report to these biochemical aspects of mechanism, nutrition, and metabolism. It has been necessary to eliminate or postpone treatment of the following subjects : the more strictly physiological or symbiotic aspects, such as metabolism of growing and resting *Azotobacter* and *Rhizobium* cells, alcoholic fermentation by legume roots and nodules, oxygen pressure functions, energy requirements, carbohydrate (-nitrogen) supply, strain variation, infective ability, host plant specificity, cross-inoculation, symbiotic promiscuity, serology, mixed cultures, bacterial antagonism, the rhizosphere, bacterial entrance and nodule formation, nodulated nonlegumes, nonnodulated legumes ; morphology, i.e., life cycles, dissociation, filterable forms, bacteriophage ; cytology ; histology ; nomenclature ; economic and agronomic aspects, viz., soil fertility, mixed cropping, and natural, artificial, and commercial inoculation ; and fixation by various new or miscellaneous biological agents.

Particular attention may be called at the outset to the splendid new book by P. W. Wilson (5), which contains extended discussion of the foregoing excluded topics, and of many other topics that can be but mentioned here. Earlier reviews, written from varying points of view during the past twenty years, may be listed : Jensen ; Wilson & Sarles ; Stephenson ; Nicol ; Gmelin ; Uspensky ; Allison (2) ; Blom ; Stapp (1) ; Giöbel ; Bonazzi ; and Greaves (2). Gmelin's *Handbuch* lists thousands of carefully classified references covering with near completeness the previous hundred years. Reviews confined almost exclusively to *Azotobacter* are those by Burk (3, 4) ; Stapp & Ruschmann ; and Greaves (1) ; and to legume symbiosis, those by Wilson (3, 4) ; Virtanen (5) ; Fred, Baldwin & McCoy ; Müller & Stapp ; and Greaves (2, Chap. 24). The Wilson & Sarles review contains twenty-seven condensed summary tables of recent quantitative data available on root

nodule bacteria, and is indispensable to the specialist in the field. Very brief accounts of nitrogen fixation have appeared in earlier volumes of the *Annual Review of Biochemistry.*[2]

MECHANISM

The effects of different gases.—Probably the most intimate information that we possess on the mechanism of fixation, and in particular on the nature of the first crucial step involved, is derived from studies of the effects of different pressures of various gases. The earlier kinetic studies of Meyerhof & Burk; Burk (1, 2, 3); and Lineweaver, Burk & Deming had clearly established that *Azotobacter* contains a material, named nitrogenase, which combines reversibly with nitrogen gas, at pressures just below atmospheric, in the simple hyperbolic manner represented by the ordinary Langmuir isotherm or Michaelis-Menton equation. During the past five years P. W. Wilson and colleagues have confirmed and greatly extended these findings for symbiotic nitrogen fixation, with further modifying work on *Azotobacter* to be indicated shortly. Varying the nitrogen pressure over red clover plants grown in association with *Rhizobium trifolii* from 0.04 to 1.56 atmospheres, Wilson (1) obtained data which upon subjection to statistical analysis indicated that above a nitrogen pressure of 0.1 to 0.15 atm., nitrogen fixation was essentially independent of pressure of nitrogen, whereas below 0.1 atmosphere the nitrogen fixation decreased rapidly. The growth of plants supplied with combined nitrogen showed no measurable dependence upon the partial pressure of nitrogen, demonstrating thereby that the pressure function with free nitrogen was associated with the nitrogen fixation process rather than with general plant growth. The Michaelis constant (K_{N_2}), or pressure for half maximum velocity of symbiotic nitrogen fixation, was estimated to be about 0.05 atm. of nitrogen. Partial pressures of helium and argon up to 0.4 atm. exerted no measurable effect on growth either in free or fixed nitrogen.

Since molecular oxygen was involved in certain suggested mechanisms of nitrogen fixation, Wilson & Fred investigated the influence of the partial pressure of oxygen on symbiotic fixation. At a pressure of oxygen greater than 0.1 atm. and less than 0.4 atm., uptake of either free or combined nitrogen was largely independent of the oxygen pressure. At pressures above 0.4 atm. there was a marked de-

[2] *Ann. Rev. Biochem.,* **9,** 513–15 (1940); **7,** 508 (1938); **6,** 597–99 (1937); **5,** 565 (1936); **4,** 611–12 (1935); **3,** 522 (1934); **2,** 491 (1933); **1,** 647–50 (1932).

crease in the uptake of both free and combined nitrogen; apparently this was associated with increased respiration and a resultant decrease in the carbohydrate reserve of the plant. At pressures below 0.1 atm. the entire plant metabolism appeared to be decreased, almost certainly due to limitation of oxygen diffusion. Since all responses to changes in the pressure of oxygen were essentially the same, whether free or combined nitrogen was supplied, it appeared that oxygen played no specific role in the symbiotic nitrogen-fixation process, as had also been indicated earlier by Burk (1) and by Burk & Lineweaver (1) for *Azotobacter*.

When hydrogen was used to replace nitrogen or oxygen during studies on the nitrogen and oxygen pressure functions, it was observed that nitrogen fixation was inhibited. Wilson & Umbreit (2) and Umbreit & Wilson established that this inhibition by hydrogen was specific for the nitrogen fixation process, i.e., hydrogen inhibited the growth of plants using free nitrogen but not of those supplied with combined nitrogen. Hydrogen caused a statistically significant decrease both in total nitrogen fixation and in percentage nitrogen content of the plants. The observed inhibition could not be attributed to impurities in the hydrogen, for carefully purified hydrogen, and hydrogen prepared from a variety of sources, induced quantitatively the same inhibition. Wilson, Umbreit & Lee extended these experiments and demonstrated that hydrogen inhibited the rate of nitrogen fixation throughout the entire growth period. Transfer of the plants, at any stage of growth, from an atmosphere containing hydrogen to air, quickly reversed the hydrogen inhibition. The approximately first order velocity constants of nitrogen assimilation observed were significantly decreased for the assimilation of free nitrogen in the presence of hydrogen, but were independent of hydrogen when combined nitrogen was supplied. Wilson (4, 5) showed that the action of hydrogen depended upon the pressures of both hydrogen and of nitrogen competitively, in accordance with the equation for the simplest type of competitive inhibition,

$$1/v = \{K_{N_2}[1 + (H_2)/K_{H_2}]/V_{max}\}/(N_2) + 1/V_{max},$$

whereby at constant (H_2) a plot of $1/v$ against $1/(N_2)$ yields a straight line of intercept $1/V_{max}$ and slope given by the expression in braces; when (H_2) is zero the slope becomes K_{N_2}/V_{max}, as in the ordinary Michaelis-Menton equation; and when (N_2) is kept constant $1/v$ is

proportional to (H_2). K_{H_2} was found by two different procedures to be a hydrogen pressure of about 0.15 atm.

Wyss & Wilson have reported that essentially all of the above findings on inhibition of symbiotic fixation by hydrogen hold also for fixation by *Azotobacter,* and in further collaboration with them the reviewers have obtained confirmation of these important observations, which obviously introduce great unity into our conception of the processes of fixation in *Azotobacter* and in legume symbiosis. Acceptance of these new findings necessitates a reinterpretation of the hyperbolic function obtained in the experiments of Lineweaver, Burk & Deming several years ago. In these experiments, conducted at constant pressure of oxygen, $(O_2) = 0.2$ atm., the hydrogen had been regarded as an inert gas, and its pressure had been varied inversely with the nitrogen pressure, (N_2), in order to maintain a constant total pressure of 1 atm., i.e., $(N_2) + (H_2) = 0.8$ atm. Inserting $[0.8 - (N_2)]$ for (H_2) in the foregoing equation for competitive inhibition yields still another rectangular hyperbola,

$$1/v = [(K_{N_2}K_{H_2} + 0.8\, K_{N_2})/V_{max}K_{H_2}]/(N_2) + [(K_{H_2} - K_{N_2})/V_{max}K_{H_2}],$$

with slope and intercept now being the respective bracketed expressions [Burk (5)]. The expression "slope/intercept" is $(K_{N_2}K_{H_2} + 0.8\, K_{N_2})/K_{H_2} - K_{N_2})$, instead of simply K_{N_2} as regarded formerly. Setting this expression equal to the accurately observed value of 0.2 atm. nitrogen pressure, invariably obtained in the case of *Azotobacter,* yields $K_{H_2}/K_{N_2} = 5\, (1 + K_{H_2})$, i.e., the ratio of the dissociation constants of hydrogen and nitrogen for the enzyme is at least 5 and probably (setting K_{H_2} as no greater than 0.4 atm. of hydrogen) not greater than 7. This value is of the same order, but a little larger than the ratio obtained in the symbiotic experiments carried out at the University of Wisconsin and conducted at constant pressures of either hydrogen or nitrogen, where, from the constants reported above, $K_{H_2}/K_{N_2} = 0.15/0.05 = 3$. This apparently smaller ratio implies that somewhat smaller pressures of hydrogen are required for a given inhibition of fixation in symbiosis than in *Azotobacter,* and further support for this possibility is derivable from the particular experiments of Wilson, Umbreit & Lee in which $[(N_2) + (H_2)]$ was kept constant just as in the *Azotobacter* experiments; using the analytical slope/intercept method outlined above yields a value of K_{H_2}/K_{N_2} even a little smaller than 3. On the other hand, whereas the slope/intercept

method gives of itself neither K_{H_2} nor K_{N_2}, but only the ratio of the two, this method applied to the short-time experiments with *Azotobacter* possibly offers a more accurate determination of the ratio than do the long-time symbiotic experiments, which as performed yielded a value for the ratio from the independent determinations of K_{H_2} and K_{N_2}. Very possibly K_{N_2} in these more difficultly controlled symbiotic experiments is in reality 0.02 to 0.03, instead of 0.05 as assigned, or K_{H_2} is even somewhat greater than 0.15, in which cases the symbiotic and nonsymbiotic ratio values might be identical. Be all this as it may, exact agreement is not necessary here of course; one recalls the well-known analogous case of carbon monoxide inhibition of the *Atmungsferment*, where the ratio K_{CO}/K_{O_2} varies between two and eight (though scarcely more) in the respiration of yeasts and other organisms.

Of considerable interest is the nature of the nitrogen and hydrogen linkages with nitrogenase. In view of the high values of K_{N_2} and K_{H_2} (each approaching an order of 1 atm.) and of their probable independence of temperature [negligible heat of dissociation (Lineweaver, Burk & Deming)], it would seem best to regard the affinities as representing not chemical activated adsorption but mainly physical van der Waals forces [Burk (5)]. Such physical forces are correlated fundamentally with heats of liquefaction and dielectric constants, and for different gases are very roughly proportional to the square roots of the boiling points, in the sense that the same series of physical forces are involved in separating molecules from themselves as from some surface or loose combination. For hydrogen and nitrogen, with boiling points of about 20° and 78° Abs., the boiling point rule would call for an affinity ratio of about half (4.4/8.9), or $K_{H_2}/K_{N_2} = 2$, which happens to be of the order experimentally observed.

One might argue that, having found the high value for K_{N_2}, and its temperature independence, one should have predicted hydrogen inhibition of nitrogen fixation before experimentally observing it. Prompted by this retrospective reflection, one might venture now to predict that other gases—helium, neon, carbon monoxide, argon, oxygen, krypton, xenon, and carbon dioxide (B.P., 6°, 34°, 81°, 87°, 90°, 121°, 164°, and 194° Abs., respectively)— would also specifically inhibit fixation, although to be sure pressures up to some 10 atm. might be required, and in the cases of oxygen, carbon dioxide, and possibly carbon monoxide, other physiological considerations might interfere with observation. No inhibition of fixation has been ob-

served with helium and argon, but the maximum pressures employed, about half an atmosphere, may not have been quite high enough. By the boiling point rule helium should inhibit less than hydrogen, and this is certainly the case. On the other hand, argon and oxygen, etc., should by strict application of the rule inhibit more than hydrogen, but the rule is after all only very approximate for the higher and nearly identical boiling points. At any rate, further experimentation on all these gases is, academically at least, still in order. The situation regarding carbon monoxide is obscure: Wilson (4) had reported that 0.1 atm. of carbon monoxide almost completely inhibited symbiotic fixation, but that much higher pressures inhibited fixed nitrogen assimilation also; neither mention nor extension of these facts is given in his later book (5). Burk (5) has definitely established that growth of *Azotobacter*, in both free and fixed nitrogen, requires, as in the case of most if not all other bacteria and higher forms, a minimum concentration of carbon dioxide; inhibition of *Azotobacter* growth by too low pressures of carbon dioxide (0.05 per cent or less) is readily observed in a Warburg apparatus by maintaining too effective absorption of respiration carbon dioxide in the alkali, with very dilute cultures. Carbon dioxide at about 1 per cent or more lowers (reversibly) the concentration range over which nitrite is toxic for respiration and growth.

In an unsuccessful attempt to measure directly, with the Van Slyke-Neill extraction chamber, the small quantity of nitrogen hyperbolically combined with nitrogenase in *Azotobacter*, Lineweaver (1, 2) obtained typical, linear solubility curves (Henry's law) for nitrogen obtainable by vacuum extraction of aqueous suspensions of *Azotobacter*, legume bacteria, and yeast cells equilibrated with different pressures of nitrogen at about 1 to 7° C. Nitrogen and argon were taken up reversibly by dried *Azotobacter* cells, especially when powdered. Much more was taken up at —183° C., where the isotherm was not linear, than at 0° C., where the uptake was still linear. This is very definitely indicative of van der Waals adsorption by the dry cells, though likely of no direct relation to the hyperbolic nitrogenase uptake.

Isotopic approach.—The availability of isotopes and their use as tracers has opened a new approach to the study of biological nitrogen fixation. Ruben, Hassid & Kamen exposed tops of barley plants to purified radioactive nitrogen, N^{13}, for twenty minutes, and then subjected the tops to a hot alcohol extraction; the extract was next boiled

in a stream of air. An extract from a control plant, killed by boiling water, showed no radioactivity whereas the extract from the live plant contained small amounts of N^{13}. As the authors stated, the evidence indicates fixation of nitrogen, but the possibility of exchange has not been eliminated, and more details and control experiments are needed for the data to be convincing. The short half life (10.5 minutes) of the radioactive N^{13}, limits its applicability and attractiveness as a tracer element; and in addition it is difficult or impossible to check for the presence of exchange reactions. The experiments show, in any event, a great difference in uptake of N^{13} by living and dead higher green plant material, whether by fixation, adsorption, exchange, or from still unsuspected fixed nitrogen impurity.

The limitations of the radioactive isotope, N^{13}, are not shared by the heavy, nonradioactive isotope, N^{15}. Not only is this isotope, N^{15}, stable, but in addition exchange reactions can be checked readily by employing a nonequilibrium mixture of the molecular species of masses 28, 29, and 30. The initial nonequilibrium condition is maintained in the absence of exchange reactions, but any exchange is apparent in a shift toward equilibrium. Burris & Miller used *Azotobacter vinelandii* in an examination of the applicability of N^{15} as a tracer in biological nitrogen fixation. After a period of fixation in air the culture was transferred to an atmosphere containing an excess of N^{15}. They observed no selective action in the fixation of N^{15} and N^{14}, no apparent exchange between molecular nitrogen and fixed nitrogen of the culture, and no exchange at the seat of fixation. They considered it unlikely that exchange reactions would interfere with the use of N^{15} as a tracer for studies of biological nitrogen fixation. As well as serving to trace the path of nitrogen and to follow the kinetics and energetics of fixation, N^{15} will prove useful in testing alleged fixation by questionable biological agents.

Excretion of nitrogen: symbiotic.—Recent investigations have revealed many interesting facts of practical and theoretical significance concerning the excretion of nitrogenous substances by the roots of leguminous plants. Virtanen (5) has traced his extensive work on excretion beginning with observations of the phenomenon in 1927 at Helsinki. In addition to Virtanen's review (5) other recent papers on the subject from his laboratory include those by Virtanen (1, 2, 4); and in great logical detail (6); Virtanen & v. Hausen; Virtanen, v. Hausen & Laine; Virtanen & Laine (6), and in great experimental detail (8); Virtanen, Laine & v. Hausen; Virtanen, Saastamoinen &

Laine; and Virtanen & Torniainen (2). In these publications Virtanen and co-workers have established beyond question that under the widely varied and studied experimental conditions used by them leguminous plants may, and almost invariably do, excrete into the soil considerable amounts of the nitrogen fixed from the atmosphere. The amounts excreted are greater than can be accounted for by sloughing-off of nodules, and excretion frequently occurs early in the life of the plant before appreciable decay or sloughing-off would be anticipated. The objection that nonsymbiotic nitrogen fixation is responsible for the observed effect is clearly answered by experiments conducted by Virtanen under bacteriologically controlled conditions. Virtanen reports that the nitrogen is excreted largely in the form of *l*-aspartic acid, and (more in older plants) its decarboxylation product, β-alanine [Virtanen & Laine (3, 5); Virtanen, Laine & Rintala]; it is also claimed that excretion of 1 to 2 per cent of oximinosuccinic acid (a postulated fixation intermediate) and nitrite may also occur.

The practical and theoretical aspects of the excretion problem have stimulated a number of other workers to investigate the subject. Predominantly negative results have been obtained by Wilson & Burton; Wilson & Wyss (2); Wilson (2); Bond (3); Bond & Boyes; Engel & Roberg; Ludwig & Allison (2, 3); Trumble & Strong; and Romashev. Slight, but questionable, excretion has been observed by Shapter and by Madhok. Variable results with some positive findings have been reported in the publications of Thornton & Nicol; Demidenko & Timofeeva; Nowotnowna; Strong & Trumble; Wilson (2); Wilson & Wyss (2); Wilson & Burton; Isakova & Andrejev; Bond (2); Bjälfve; and Scholz.

Wilson (2) conducted a joint experiment with Virtanen in Helsinki and obtained some positive evidence of excretion, although the period of the year (late fall) was least favorable for markedly positive results. Subsequently Wilson & Wyss (1) were in several instances able to obtain excretion in their experiments performed at Madison. Extensive experimentation led Wilson (5) to conclude that excretion is obtained only under particular conditions, namely those providing sufficient photosynthesis to insure a fairly high rate of nitrogen fixation but without excess of carbohydrate to bind into the plant all nitrogen as it is fixed. A number of differences between the modes of plant development at Helsinki and at stations reporting negative results on excretion were listed.

Strong & Trumble, by reducing the illumination on pea plants to

two hours of sunlight per day, induced excretion, although under normal conditions at the station at Adelaide, Australia, they never observed excretion. The recent failure of Ludwig & Allison (2) to obtain excretion in tests made at Washington, D.C., under controlled conditions, including those which appeared to produce excretion at Madison, emphasizes Wilson's (5) conclusion that a technique apparently effective in establishing proper conditions for excretion in one locality may well be ineffective in inducing the result elsewhere. The conditions necessary for invariably obtaining excretion in symbiosis evidently remain to be defined, granted that the Virtanen school has made great efforts and much progress in this direction. Likewise conclusive work needs to be done regarding excretion by plants supplied fixed nitrogen, under a variety of conditions, in order to establish whatever specificity there may be for the symbiotic excretion resulting from fixation.

Experiments on nitrogen transfer within the plant, rather than external excretion, are reported by Bond (1) and by Wilson & Umbreit (1).

Excretion of nitrogen: nonsymbiotic.—Good summaries of earlier work on extracellular nitrogen yielded by *Azotobacter* are given by Roberg and by Burk & Horner (5). In recent years excellent synthetic media, including in particular adequate molybdenum and iron, make it possible to obtain large quantities of *Azotobacter* nitrogen (20 to 50 mg. per 100 cc.) and to determine with much less qualitative ambiguity and far greater quantitative precision than heretofore the large number of kinds and amounts of nitrogen in the extracellular fraction. On the latter basis Horner & Burk (2) summarized an exhaustive and recent analysis of the major facts of interest for pure cultures of *Azotobacter,* including variables such as species, age, energy supply, added fixed nitrogen, degree of aeration, and inorganic nutrition. In general, young cultures vigorously fixing nitrogen excrete some 10 to 25 per cent of the nitrogen into the surrounding medium. This extracellular nitrogen is quite a heterogeneous mixture; about two thirds is precipitable by lead acetate, one third by phosphotungstic acid, one fifth by aluminum sulfate and still less by trichloroacetic acid. (With old cultures the amounts for the last three precipitants increase to half, a third, and two fifths respectively.) Well over half can be dialyzed through cellophane. Amino nitrogen seldom represents more than 10 to 30 per cent of the total soluble nitrogen, and ammonia is not formed so long as readily available

oxidizable matter is present, but upon disappearance of the latter some 8 to 12 per cent of ammonia nitrogen may form (contaminants may increase the yield to over 50 per cent). Some *A. chroococcum* strains yielded oxime nitrogen (up to 1 p.p.m.), and *A. vinelandii* occasionally yielded hydroxylamine nitrogen (up to 0.05 p.p.m.), but most strains yielded neither of these (less than 0.01 p.p.m.). In old cultures with depleted carbohydrate supply the extracellular nitrogen may increase to 40 to 75 per cent of the total nitrogen, with higher proportions of the more complex types, including much protein, and corresponding decreases in amino nitrogen. Cultures supplied fixed nitrogen for nutrition will generally excrete somewhat more soluble nitrogen than nitrogen-fixing cultures, but with little real difference except qualitatively for nitrite production from nitrate, and ammonia from cultures growing on nitrate, asparagine, or urea, even in the presence of adequate carbohydrate supply (nonautolyzing conditions).

Older, less extensive or more specialized studies similar to the foregoing have been reported by Kostyschev, Ryskaltschuk & Schwetzova; Kostyschev & Scheloumova; Isakova; Roberg; Bortels (3); Winogradsky (1 to 7); Winogradsky & Winogradsky; and Burk & Horner (2, 5). Some of these, including the two latter, are partially ambiguous because the cultures used were not strictly pure. Virtanen & Laine (3) have reported specifically on aspartic acid excretion by *Azotobacter*. Oxime or hydroxylamine formation has been reported by Blom; Endres (1, 2, 3); Endres & Kaufmann; Burk & Horner (1); Virtanen & Laine (3); Suomalainen; and Virtanen (5).

Specific intermediates in nitrogen anabolism.—Apart from the physicochemical evidence concerning nitrogenase already presented there is at present no further information as to the nature of the initial product or products of nitrogen uptake, which are of greatest interest in connection with the chemical mechanism of fixation. The classical method for investigating biochemical mechanism, involving isolation of definite chemical intermediates, has offered little of value here, but concerning later stages, probably more accurately regarded as nonspecifically metabolic, the available information just described in the two previous sections on excretion is highly suggestive. On the other hand, distinction between specific initial stages and later metabolic stages has often been lost sight of. For many workers it has been psychologically irresistible to conclude upon finding some extracellular nitrogenous product that it was involved in either the initial or later anabolic rather than catabolic stages. Although a considerable

number of isolated and hypothetical compounds have been so regarded
during the past fifty years, the main controversy during the past dec-
ade has been confined to the two rival intermediates, ammonia and
hydroxylamine (or oxime). The present weight of evidence favors
the latter, but by no means offers unequivocal support.

The ammonia theory, originally advanced with tolerable evidence
by Kostyschev and co-workers, and then greatly amplified by Wino-
gradsky (1 to 7), has been heavily criticized by Burk & Horner (2,
5) ; Horner & Burk (2) ; and Roberg. It has naturally met with no
acceptance by the proponents of the hydroxylamine theory (Blom,
Endres, Virtanen), nor indeed by the field generally, according to
a reiterated reflection of Winogradsky (4, 6). In the latter very in-
teresting paper, Winogradsky has continued to present evidence sup-
porting his theory, claiming that autolyzing silica gel cultures of
Azotobacter yielded more ammonia nitrogen after disappearance of
the organic substrate than corresponded to the simultaneous loss of
total nitrogen. In Winogradsky's opinion, a highly efficient enzymic
synthesis of ammonia was involved, which accumulated slowly over
a period of months a relatively important amount of ammonia, under
conditions that might well obtain in soils or waters. Suggestive and
novel as the new experiments were, however, they did little to over-
come previous criticism [Burk & Horner (5), pp. 113–117] concern-
ing the inevitable difficulties encountered in employing the silica gel
technique—in biochemical as distinguished from agrobiological stud-
ies—with its heterogeneous and indefinite mixture of living and dead
cells, its questionable or unspecified culture purity, and the uncertain
time and uniformity of substrate disappearance on a plate as a whole
or by parts. Correlations of the time courses of nitrogen fixation and
ammonia formation with growth, and energy consumption and dis-
appearance, present formidable difficulties in determining whether the
ammonia observed must be an intermediate in fixation or derived
from nitrogen only after being built into stable cell nitrogen and then
formed in normal decomposition processes of autolysis or deamination
such as are observed in many ammonia-forming, nonnitrogen-fixing
organisms. The "excess ammonia" obtained by Winogradsky is in-
deed not large when recalculated in relation to the total nitrogen fixed;
and with due consideration of the error and uncertainty connected
with establishing the time at which the added substrate had disap-
peared—uncertain because in its later stages it is a drawn-out pro-
cess—it becomes difficult or impossible to be certain when synthesis

of cell nitrogen had ceased. In the absence of assurances as to mainte-
nance of culture purity during the several months of continued ob-
servations of plates, the rather high proportion of ammonia nitrogen,
50 per cent or more of the total nitrogen, may have been due in part
to extraneous ammonifying organisms. The efficiencies of fixation
reported by Winogradsky, when recalculated in terms of total fixed
nitrogen measured per sugar equivalent of substrate apparently added,
are in fact of an order not uncommonly observed (experimental error
being considered) and are far below those claimed by Bach, Iermo-
lieva & Stepanian in their still unconfirmed experiments with *Azoto-
bacter* press juice. Far from, in Winogradsky's words "physiological
ideas precluding a decomposition process under the given conditions,"
it seems necessary to continue to affirm, without undue discourage-
ment of future work along this line, however, that normal decompo-
sition processes appear to account for all extracellular ammonia so
far observed by any investigator; the occurrence of any ammonia,
observed or otherwise, as an essential intermediate in nitrogen fixation
by *Azotobacter*, admittedly still possible, yet awaits sufficiently critical
experiments in support.

The hydroxylamine intermediate theory, advanced early in the
decade by Blom, who obtained traces of hydroxylamine in cultures of
Azotobacter grown in nitrogen or nitrate, is now mainly supported
by the data on excretion in symbiotic fixation obtained by Virtanen
et al. The hydroxylamine is not regarded as an anabolic by-product,
nor as a catabolic product as just indicated for ammonia, nor is it re-
garded in any precise sense as an initial fixation product. It is held to
be a definite anabolic intermediate derived in a series of enzymic re-
actions from either nitrogen, nitrate, or nitrite, and continuing as the
starting point where inorganic nitrogen is introduced into the carbon
chain in amino acid synthesis: as Endres (4) has rightly pointed out,
specific assimilation products of nitrogen must eventually at some point
fall onto the path of nitrogen anabolism taken by other simple fixed
nitrogen compounds in their assimilation.

According to Virtanen *et al.,* hydroxylamine reacts with oxalacetic
acid to yield oximinosuccinic acid which on reduction yields aspartic
acid. As support for this scheme it is claimed that (*a*) the chief prod-
uct of excretion is aspartic acid or its enzymically decarboxylated
product β-alanine, (*b*) oximinosuccinic acid is excreted in small quan-
tities, (*c*) oxalacetic acid is present in the tissues of leguminous plants,
and (*d*) excised root nodules fix nitrogen if oxalacetic acid is pro-

vided [Virtanen (4, 5, 6); Virtanen & Laine (3, 4, 6, 7, 8); and Virtanen & Arhimo (1, 2, 3)]. The oxime was not found with plants given ammonia nitrogen, and Virtanen & Arhimo (3) postulate that with ammonia nitrogen nutrition the ammonia would react with oxalacetic acid to again yield aspartic acid via the imide instead of the oxime. The oxalacetic acid is derived from glucose via fumaric acid, which Virtanen & Laine (8) also claim to have isolated.

Endres & Kaufmann believed that with *Azotobacter* the unidentified oxime observed by them was not formed from hydroxylamine, since hydroxylamine itself was never found; as Horner & Burk (2) have indicated, however, whether oxime or hydroxylamine is found, if either is formed at all, depends upon the strain and other cultural and analytical conditions. Virtanen *et al.* obtained oxime and not hydroxylamine with both symbionts and *Azotobacter,* but had no logical scruple against assuming derivation of the oxime directly from hypothesized hydroxylamine. Presenting a fixation scheme similar to Virtanen's, Suomalainen introduced nitrite as preferable to hydroxylamine as the initial inorganic intermediate combining with oxalacetic acid. To this can be raised the objection that no single authenticated case of nitrite formation has ever been observed in nitrogen-fixing *Azotobacter* cultures, and Virtanen & Laine (1, 8) claim that the nitrite found in symbiotic excretion is derived from the oxime during extraction and evaporation.

Wilson (5) and Wilson & Wyss (2) regard the isolation of the oximinosuccinic acid as much more impressive evidence for the hydroxylamine theory than the other lines (*a*), (*c*), and (*d*) above, for which confirmation elsewhere is lacking. Virtanen (5) had reported as high as 0.1 per cent oxalacetic acid in pea plants grown at Helsinki, but Wyss, Burris & Wilson have been unable to detect any in leguminous plants grown in Wisconsin, although they could readily detect small added quantities. Wilson (4) and Allison, Ludwig, Hoover & Minor had no success in inducing fixation by excised root nodules by addition of oxalacetic acid. Until more extensive details of definitely positive results are reported as promised [Virtanen & Laine (8); and Virtanen (6)], the results of Virtanen & Laine (4) must be regarded as highly provisional, since the percentage increase in nitrogen by the excised nodules allegedly fixing nitrogen was of an order, for a point so important as the one involved, not definitely beyond the statistically significant error of analysis for nodule lots of the size employed. If the Virtanen school eventually prove to be right in obtaining fixation

by excised nodules with oxalacetic acid, this step will undoubtedly constitute the most significant experimental advance to date in the elucidation of the problem of symbiosis, which

"is to find out, on the one hand, the carbon compounds which the intranodular bacteria receive from the host plant in connection with the fixation of nitrogen in the root nodules, and, on the other hand, the nitrogen compounds which the host plant in its turn receives from the root nodules" (Virtanen, 6).

The hydroxylamine (oxime) theory, the pros and cons of which have recently been set forth in greatest detail by Virtanen (6), Burk (4), and Wilson & Wyss (2), has much plausible, almost irresistible, support. This support is by no means as rigid as commonly obtains in many schema in organic chemistry, nor as can be provided by studies with isotopes. It is probably of more immediate interest for nitrogen metabolism of legumes than for fixation specifically. The greatest weakness of the theory is that neither hydroxylamine nor oxime has ever been clearly shown to be an available source of nitrogen for nutrition of either symbionts or *Azotobacter* [Burk & Horner (1); Endres & Kaufmann], even at nontoxic concentrations.

Particular compounds produced by nitrogen-fixing agents.—In an effort to locate some product peculiar to the fixation process, Orcutt compared fractions of the soluble nitrogenous material in soybean plants fixing atmospheric nitrogen and soybean plants supplied with combined nitrogen. The general situation for the two types of nutrition was similar, and the only compounds which appeared to offer possible significance for fixation were those present in the basic nonamino fraction. After initiation of nitrogen fixation by the plants, the basic nonamino fraction was consistently higher than in plants supplied combined nitrogen. Fractionation of the soluble nitrogenous material from soybean nodules by Umbreit & Burris indicated the existence of a compound, in the basic nonamino fraction, which readily released ammonia under alkaline conditions. Their tentative suggestion that the compound was arginine appears to be incorrect, and further work by Umbreit [unpublished data cited by Wilson (5)] indicates that the substance may be a ureide.

Virtanen & Torniainen (1) reported the concentrations of a number of amino acids in dried pea nodules. Data concerned with the nitrogen fractions of four species of rhizobia (Umbreit & Burris) show but slight variations between species.

Cooper & Preston, and Cooper, Daker & Stacey have studied the optimum conditions for the formation of gum by *Rhizobium* and

Azotobacter, and have confirmed the earlier work of Hopkins, Peterson & Fred indicating a higher glucuronic acid content of hydrolyzed gum from clover and pea organisms than from the alfalfa strains. Both the *Rhizobium* and *Azotobacter* gums appeared to belong to the same class as those of types II and III pneumococcus specific polysaccharides, yielding glucose and uronic acid on hydrolysis. Anderson has made a detailed study of gum formation by various rhizobia and Hamilton has studied *Azotobacter* gum.

Azotobacter agilis and *A. vinelandii* are very similar in composition, both having high protein and low carbohydrate and lignin contents even when grown on agar [Greene, (2)]. *A. beijerinckii* and *A. chroococcum,* on the other hand, had high lignin and carbohydrate and low nitrogen contents. Greene found that the forms of nitrogen in the four species varied little, and arginine and lysine were the amino acids found to be present in largest amounts. Approximately 40 per cent of the nitrogen appeared in the nonbasic fraction, whereas with several species of rhizobia Umbreit & Burris reported about 60 per cent nonbasic nitrogen.

Sifferd & Anderson have demonstrated the formation of a sterol by *A. chroococcum,* which is probably a mixture, and more related to ergosterol than ordinary plant or animal sterols.

Nilsson has shown that *A. chroococcum* produces large quantities of cozymase, and also hexosediphosphate dehydrogenase and, adaptively, mannite dehydrogenase.

Evidence that nodules contain dihydroxyphenylalanine (dopa) and its oxidation products has been presented by Mothes, and by Mothes & Pietz. They believe that the substance acts in poising the oxidation-reduction potential in the nodule at a level favorable for growth and fixation by rhizobia. According to Kubo (2) the "red body" present in nodules, which Pietz described as an oxidation product of dihydroxyphenylalanine, is in fact a hemoprotein. Kubo (2) found that the hemoprotein, which he has isolated from the nodules of many leguminous species, on dissociation gives a hemin identical in crystal form with the hemin from horse hemoglobin. He reported that the hemoprotein stimulated succinate oxidation by *R. japonicum.*

The root nodule bacteria do not appear to possess an active proteolytic system. New data concerning this fact have been furnished by Virtanen & Laine (2), who observed but slight increases in soluble nitrogen during a six months' incubation period of *R. trifolii* and *R. leguminosarum* on media containing glucose and casein or yeast

extract. Berger, Johnson & Peterson found *R. trifolii* had a readily measurable but rather low peptidase activity.

Azotobacter is rich both in number and content of metabolic enzymes, including, besides nitrogenase, the spectroscopically observable *Atmungsferment,* cytochromes *b* and *c*, catalase, urease, flavocompounds, numerous dehydrogenases, malonate decarboxylase, and glutathione, as reported by many investigators during the decade.

The extensive current researches on auxins have touched the subject of symbiotic nitrogen fixation with the suggestion that auxins may initiate the formation of root nodules. Link reported that bean nodules contain 3-indoleacetic acid and other auxones, and that these substances may incite nodulation. In extending these studies Link & Eggers found that nodules of beans and peas had different auxones and a higher auxone content than roots grown on sterile substrates. Thimann also suggested a possible role for auxins in nodule formation, and cited the production of 3-indoleacetic acid by pure cultures of rhizobia as supporting evidence. Georgi & Beguin, however, believe little significance can be attached to this observation, for they found that the soil organism *B. radiobacter* produced auxin at a more rapid rate than the root nodule bacteria. Evidence that auxins are really critical in the formation of the highly differentiated tissue of root nodules is as yet merely suggestive.

NUTRITION

The nutrition of *Azotobacter* has been the subject of an untold number of papers because of its widespread agronomic interest; because the organism will grow excellently in a strictly synthetic medium without addition of organic growth factors; and, during the last decade especially, because of the hope that it might be possible to discover aspects specific for fixation as distinguished from nitrogen nutrition generally. Owing to the prevailing lack of knowledge of the coexisting needs of molybdenum, iron, and calcium, until some five years ago, and to inadequately maintained buffering of the medium, and to the rather misleading type of ideas disseminated by F. Löhnis and collaborators some twenty years ago as to the large variety of forms that may constitute the cell morphology of a pure culture of *Azotobacter,* a great deal of the nutrition literature is so conflicting and uninterpretable that it will not be fully resolved until certain aspects have been investigated anew in the light of modern knowledge, with media essentially optimum in every known constituent and cultures of con-

tinually proven purity. The two latter desiderata, simple as they appear, have not been generally attained until the last few years. Before this investigators were commonly resigned to obtaining fixations of only about 5 mg. of nitrogen per 100 cc. unless large quantities of soil humate or similar material were added. Now with adequate supplies of molybdenum, iron, and calcium, fixations of 20 to 50 mg. of nitrogen per 100 cc. should be expected in a few days on media containing 1 to 3 per cent sugar. With the lack of one or the other of these components usually obtaining, and contamination often suspectable (especially in cultures with fixed nitrogen added), much work on *Azotobacter* nutrition before 1935 must be interpreted with due caution, or even rejected. For a decade Winogradsky has been emphasizing the importance of contaminants developing, in old cultures particularly.

Peptone media purity control.—Sugar-free peptone media, in which *Azotobacter* will make very little growth, but common contaminants of *Azotobacter* make a good growth, were widely used to test for purity of *Azotobacter* cultures by the early workers in the first five years of the century (Beijerinck, Gerlach, Vogel, Koch, Heinze, Lipman), but for no evident reason they were almost never used until a few years ago when their use was revived by Burk (5), Winogradsky (5), and Horner & Burk (2). In filtered-clear, sugar-free mineral media containing 1 per cent bactopeptone and 0.1 per cent meat extract, *Azotobacter* will attain only 20 to 50 millions per cc., but common even if not all contaminants will grow up to several orders greater. A drop of *Azotobacter* culture containing one contaminant per hundred organisms, inoculated into the peptone medium, will within a day or so yield a converse ratio of one *Azotobacter* organism per hundreds of contaminant organism, detected often by a pH increase, almost invariably evident visually or turbidimetrically, and certainly evident with the obligatory microscopic control. It is recommended that every culture of presumed purity be tested routinely with peptone tubes, whatever occasional plating-out procedures are followed. Cultures of purity tested by peptone and plating-out have shown almost none of the very numerous morphological forms claimed by Löhnis, when grown on various media over extended periods of time, in the experience of the reviewer (D. B.), and in line with the findings of Lewis and of Roberg who report finding no organisms less than 0.75μ in diameter.

Fixed nitrogen.—Comparatively little work has been done recently

on utilization of fixed nitrogen by *Azotobacter,* and reports are rather conflicting or indecisive for reasons just indicated [Fuller & Rettger; Thompson; Burk & Horner (3, 4); Aso, Migita & Ihda; Greaves, Jones & Anderson]. Older work needs to be repeated with adequate supplies of trace elements (iron, molybdenum, vanadium, tungsten, etc.). It seems probable that *Azotobacter* uses, in addition to nitrate, nitrite, ammonia, and urea, only a few other simple organic nitrogen compounds, including asparagine (with adequate molybdenum) and possibly arginine; whether any considerable number of amino acids is utilized, even after prolonged incubation, is questionable. With adequate supplies of molybdenum present, nitrogen gas can often be utilized simultaneously with, and even preferably to, any nitrogen compound except possibly ammonia; temperature and the strain of organism affect the quantitative relations. Hydroxylamine and hydrazine are not utilized [Burk & Horner (1)]. Virtanen (6) makes the important claim that legumes utilize aspartic acid in preference to all forms of fixed nitrogen, and that nonlegumes (wheat, barley) use it scarcely at all, a claim of great significance for the hydroxylamine (oxime) theory of legume nitrogen nutrition.

Inorganic nutrition.—The major inorganic elements and concentrations thereof needed by *Azotobacter* were worked out by Krzemieniewska and reported in a classical paper (1910) that is so generally overlooked as to warrant citation here. Work of the past decade has been concerned mainly with the minor or trace elements, and is reviewed in concise detail by Pirschle, both in relation to nitrogen-fixing and other organisms.

Following the initial lead of Bortels (1), many investigators have shown the need of molybdenum in growth by *Azotobacter* [Bortels (2, 3, 4, 6, 7, 8, 9); Burk & Lineweaver (2); Burk (3, 4); Burk & Horner (3, 4, 6); Horner, Burk & Allison; Birch-Hirschfeld; Schröder; Konishi & Tsuge; Kluyver & van Reenen; van Niel; Baier; Bassalik & Neugebauer (2); Rippel; Flieg; Krzemieniewski & Kovats; and Kovats]; by legume symbionts [Bortels (2, 5); Dmitriev; Obratzova *et al.*]; by nitrogen-fixing blue-green algae and other bacteria [Bortels (2, 3); Stapp (2)]; by *Aspergillus* [Steinberg (1, 2)]; and by higher green plants (Arnon & Stout; De Rose *et al.*). Concentrations as low as 10^{-12} M molybdenum may give measurable effects in very dilute cultures of *Azotobacter* [Burk (3)], but with heavy cultures upwards of 1 p.p.m. will be needed for maximum effect [Bortels (3, 8); Krzemieniewski & Kovats; Horner, Burk &

Allison]. Vanadium usually can replace molybdenum in fixation, but is definitely less effective [Bortels (3, 4, 5, 8); Burk (3); Burk & Horner (3); Horner, Burk & Allison]; Bernheim & Bernheim have shown that vanadium markedly increases oxygen uptake by rat or guinea pig liver suspension but that molybdenum plays no similar role. With the possible occasional exception of tungsten, molybdenum and vanadium cannot be replaced by any elements in long lists tested by Bortels (1); Burk (3); Flieg; Steinberg (1); Krzemieniewski & Kovats; Arnon & Stout. An effect of tungsten in replacing molyb-denum or vanadium has been indicated by Bortels (1); Schröder; Krzemieniewski & Kovats; and Horner, Burk & Allison, but only at quite high concentrations of tungsten, and an impurity of less than 0.1 per cent molybdenum in the tungsten would explain the effect. Likewise the need of *Azotobacter* for other trace elements similar to molybdenum or vanadium or tungsten (e.g., zinc, copper, silicon, manganese, iodine) has been variously claimed [Schröder, Greaves (3), Bortels (8)] but without convincing certainty.

The growth increases obtained with molybdenum range from zero to twenty-fold or more, depending upon a considerable number of readily demonstrable factors, and are greater (*a*) the smaller the impurity in the medium, especially in the sugar; (*b*) the smaller the inoculum; (*c*) in stagnant than in aerated or shaken cultures, i.e., using the Warburg technique (probably a function of the heaviness of culture and the extent of oxygen supply); (*d*) in older than in younger cultures; (*e*) with increasing temperatures, even well be-yond the optimum; (*f*) with *A. chroococcum* than with *A. vinelandii* or *A. agilis,* generally; and (*g*) with nitrogen than with nitrate or asparagine. Molybdenum ordinarily increases the efficiency of growth and nitrogen assimilation several fold, and with the other conditions in the medium optimal, up to 20 mg. of nitrogen fixed per gram of sugar decomposed should be obtained, and even greater quantities of ammonia or nitrate assimilated due to greater yields of extracellu-lar nitrogen formed. Such efficiencies were seldom if ever obtained, except at very low pressures of oxygen [Meyerhof & Burk; Burk (1, 2)] until the role of molybdenum in *Azotobacter* nutrition became known.

Bortels (3) reported that in stagnant Erlenmeyer-flask cultures of *Azotobacter,* maintained in air, assimilation of nitrate, asparagine, and ammonia was increased by molybdenum; asparagine by vana-dium and possibly by tungsten; and nitrate, and perhaps ammonia,

by tungsten. Burk & Horner (6) have confirmed these results for similar cultures maintained in 20 per cent oxygen (with or without hydrogen up to 1 atm. total pressure), except for obtaining no effect of molybdenum or tungsten on ammonia assimilation under conditions where any possible influence of nitrogen gas was excluded. The effect of molybdenum and probably of vanadium on asparagine assimilation opposed the view that these trace elements are concerned only in reduction, e.g., reduction of nitrogen or nitrate. To establish that molybdenum and tungsten, as well as vanadium, have no effect on ammonia assimilation, might indicate that the function of these elements is to convert other nitrogen compounds to ammonia. On the other hand, absence of an effect may mean merely that molybdenum present in the medium as an impurity is effective at relatively lower concentrations for ammonia assimilation as compared with all other nitrogen compounds, where the growth velocities are smaller. Much more work, with a variety of fixed nitrogen compounds, is needed. In the experiments of Burk & Horner hydrogen had no measurable effect on growth in any fixed nitrogen compounds studied.

Uncertainties earlier in the decade in the papers of Burk, Lineweaver & Horner (1), Bassalik & Neugebauer (1, 2, 3), Bortels (1), Greaves (3), and Horner & Burk (1) regarding the relative importance and independence of iron, molybdenum, and soil humate in *Azotobacter* nutrition have been fully clarified by the papers of Krzemieniewski & Kovats; Kovats; Bortels (3, 8); and finally by Horner, Burk & Allison. The beneficial effect of soil humate is due to either or both iron and molybdenum, depending upon circumstances, and can be equalled or surpassed by the simultaneous addition of iron and molybdenum at a few p.p.m. The beneficial effects of either one added alone are decreased or eliminated if the other is sufficiently limiting. This chapter in *Azotobacter* nutrition, in reality extending back thirty years, is one of the most satisfactorily resolved, and is an excellent model for studies with other organisms. The stimulating action of 0.1 per cent agar, and similar colloids, has been reported by Rippel; Baier; Virtanen (3); Sullivan; and Horner, Burk & Allison, and is evidently not particularly related to iron or molybdenum but probably to oxygen supply. Burk (5) has found that the agar effect is eliminated by using adequately shaken or aerated cultures. Greaves & Anderson have described the qualitative and quantitative sulfur requirements of *Azotobacter* in media without added molybdenum. Stapp & Bortels, and Bortels (6, 7, 9) claim

that *Azotobacter* development is markedly influenced by meteorological conditions, but it is probable that this *Wetterfaktor* will not be substantiated by others for at least some time to come.

Definitely contrary to earlier views or implications of Burk & Lineweaver (2); Burk (3); Horner & Burk (1); Schröder; and Bortels (3), the quantities of calcium required to obtain maximum or given submaximum growths of *Azotobacter* are, according to Burk & Horner (6), practically the same in free as in various forms of fixed nitrogen (nitrate, nitrite, ammonia, asparagine) when adequate iron and molybdenum, etc., are present. This is true, moreover, at all physiological concentrations of magnesium. Oxalate, which combines with and removes calcium, is equally inhibitory to growth in free and fixed nitrogen. There now seems to be little ground for believing that calcium (or strontium) is specifically required in nitrogen assimilation. The role of calcium in legume nutrition has been described by Albrecht & McCalla; Albrecht; and Horner. The role of phosphorus has been considered by Hutchings, and of mineral nutrition in general by Wilson (3).

It thus appears that although all nitrogen-fixing organisms so far tested require molybdenum (or vanadium), iron, and calcium (or strontium), in no case—regardless of earlier indications—can it now be regarded as probable that these elements are specifically required in fixation as distinguished from general fixed nitrogen assimilation. Various claims for specificity have been made at one time or another— often by the reviewer (D. B.)—but as is so frequently the case with specificities claimed in science generally, accumulation of later more extensive, varied, and controlled data has eliminated the seemingly unique qualitative aspects of the basic observations. Categorically and without detail, the same may be said regarding the specific hydroxylamine inhibition of fixation claimed by Endres (2) and Kubo (1) but not confirmed by Burk (5); and likewise the limiting pH 6.0 specificity claimed for *Azotobacter* by Burk & Lineweaver (2), and by Burk, Lineweaver & Horner (2), and now probably better understood as originally interpreted on a respiration basis by Burk, Horner & Lineweaver. In all these cases, certain quantitative differences in behavior between free and fixed forms of nitrogen may be observed under some particular conditions, to be sure, but for the time being these cases are probably better referred to secondary differences in growth velocities, markedly suboptimal conditions possibly obtaining, or in some instances even to unsuspected culture impurity. The only

qualitative fixation specificity that can be regarded as definitely established at present is hydrogen inhibition, and even this is probably essentially physical rather than chemical.

Organic growth factors.—With the very heavy growths now obtainable in synthetic media containing adequate iron and molybdenum it has become more certain than ever that *Azotobacter* probably requires no organic material of colloidal or vitamin-like nature essential for growth. Where claims otherwise have been made, it is not unlikely that the synthetic medium employed was inorganically deficient or inadequate [cf. Greaves (4) for vitamin-B action in Ashby's medium].

Many strains of rhizobia, however, have long been known to require or markedly benefit by complex plant or animal extracts added to the medium [cf. Allison (1); Fred, Baldwin & McCoy]. The chief factor in these extracts was finally shown by Allison, Hoover & Burk to be a coenzyme, designated by them as coenzyme R. It was heat-stable, dialyzable, separable from organisms, and affected respiration of rhizobia even more directly than growth (cell number or turbidity increase). Establishment of this factor as essential in respiration and hence growth has served to resolve a great many of the major, hitherto puzzling aspects of legume bacteria physiology [Allison & Hoover (1 to 5); Allison & Minor]. Confirmation of its role was readily obtained by McBurney, Bollen & Williams; Thorne & Walker (2, 3); Clark; and Nilsson, Bjälfve & Burström (1 to 5). Doubt or denial of its role persisted in the literature for some time [Thorne & Walker (1, 4, 5); Thorne; Steinberg (1); West & Wilson (2, 4)], but this is now evidently ascribable to the use of media or inocula containing unsuspected or unrecognized coenzyme R or foreign organisms; confusion with inorganic trace elements, iron requirements, reducing agents, or oxidation-reduction potential effects; or failure to distinguish between full heavy growths of prime interest, and small growths of minor importance for the question of essential dietary growth factors, as ordinarily understood. The conclusion of West & Wilson (4) appearing as late as 1939, that *"Rhizobium trifolii* synthesizes all the organic substances essential for its growth from the simple ingredients of a properly reduced carbohydrate mineral-salts medium," is, in the opinion of the reviewer (D. B.), inadmissable; for there is probably no organism where the essentiality of, not mere stimulation by, an organic growth factor necessarily provided in the nutrient medium can be demonstrated more definitely and clearly than in the case of coenzyme R for a large variety of strains of rhizobia, in which ratios in amounts

of growth of as much as 500 to 1 are obtainable in the presence and near absence, respectively, of coenzyme R; to claim that no growth factor is involved here is thus to be in the untenable logical position of denying the existence of growth factors generally.

As indicated in the first paper of Allison, Hoover & Burk, coenzyme R is required for good or maximum growth by most of the fast-growing rhizobia (clover, alfalfa, bean, pea), with little response by the slow and poor growers, soybean, cowpea, and lupine. Later work by Allison & Minor has shown that two *Dalea,* one *Coronilla,* and one alfalfa strain out of thirty tested actually synthesize coenzyme R, although the alfalfa strain not enough to cover its needs. *Azotobacter* and the *Dalea* organism synthesize greater, in general far greater, quantities of coenzyme R than any other organism so far reported upon, and most of the coenzyme R so synthesized is liberated into the medium, both by vital secretion and autolysis. This great production of coenzyme R by *Azotobacter* may account in part for its beneficial action in some soils. J. Wilson & P. W. Wilson report in a very recent abstract that the majority of fast-growing *Rhizobium* strains can be continuously transferred on a synthetic medium from which biotin (coenzyme R) has been eliminated with the production of one tenth the growth when this factor is added, but until the experimental details are available the implications of the result must —in the opinion of the reviewer (D. B.)—be regarded as very questionable in view of both the truly great difficulty of eliminating coenzyme R from media, and, still more important, the long-standing results of Allison and co-workers who have obtained much greater reductions in growth ($\frac{1}{50}$ to $\frac{1}{500}$ optimal) upon extensive, but probably never quite complete, elimination of coenzyme R from sugar-mineral medium.

Allison, Hoover & Burk showed in their initial publication that coenzyme R is not identical with the complex, bios, and left open the question of its relationship to components thereof. It remained for Nilsson, Bjälfve & Burström (6, 7), and very shortly thereafter West & Wilson (3, 5), to show its virtual identity with Bios IIB, or biotin. Still further confirmation was later provided by György, Melville, Burk & du Vigneaud, who concluded that these two substances were also identical with vitamin H, a conclusion that was further established by du Vigneaud, Melville, György & Rose, and by György, Rose, Hofmann, Melville & du Vigneaud. The establishment of the identity of biotin, coenzyme R, and vitamin H is obviously of great

significance for connecting plant and animal vital economy, the more so in view of the already demonstrated role of coenzyme R in respiration and hence probably in fundamental intermediate metabolism, and also in view of the many directions which research on biotin has taken in 1940, since its connection with animal metabolism has become known. György, Rose, Hofmann, Melville & du Vigneaud have obtained a crystalline biotin methyl ester with a melting point of 18° C. higher than that reported by Kögl & Tönnis, and of several-fold greater biotin and vitamin-H activity. With this pure substance the reviewers have obtained typical coenzyme-R responses with *Rhizobium meliloti* Wisconsin Strain Nos. 100 ("good"), 101 ("variable"), and 131 ("low gum") and *R. trifolii* Strain Nos. 202 ("poor"), 205 ("good"), and 209 ("good"), with half-maximum responses of Strain Nos. 209 and 101 being obtained at 0.00001 μg. per cc. of growth medium, and 95 to 100 per cent maximum growth at 0.0001 μg. per cc., which are approximately ten-fold lower concentrations than shown by Fleischmann Strain 139 distiller's top yeast, indicating that rhizobia have a lower requirement for biotin than this (typical) yeast, and, correspondingly, are a more sensitive organism for assay purposes. In spite of the demonstrated identity of biotin, coenzyme R, and vitamin H (and likewise *Clostridium* factor, etc.) it seems desirable that the two latter terms should be retained, in the same way as have the corresponding terms vitamin A, B_1, B_2, C, D, etc. Such terms connote the biological activity of these substances whereas the strictly chemical names do not. Often, for any one vitamin, a variety of natural or synthetic derivatives and substitutes exist, no one of which can exclusively be called the respective vitamin.

Other known nutrient growth factors for rhizobia apart from coenzyme R may be said to exist only for a few strains, or by virtue of an almost unjustifiable extension of terminology. Nilsson, Bjälfve & Burström (1 to 7), and Bjälfve, Nilsson & Burström reported that three out of six clover strains required vitamin-B_1 addition for good or maximum growth. No such effect was observed with five bacterial strains from pea, two from bean, three from alfalfa, two from lupine, two from cowpea, one from soybean, and two from lotus. Thus only three positive responses were obtained from the twenty-five strains examined. Laird & West reported negative results for addition of vitamin B_1. West & Wilson (2, 4) report that washed organisms of *R. trifolii* yielded negligible growth until riboflavin and thiamin were added to the medium. In the opinion of the reviewer (D. B.) the term

"growth factor" in the accepted sense would scarcely seem appropriate here, since treated and not normal cells were involved. The washing may have removed not only extracellular metabolic products as assumed by West & Wilson but also intracellular constituents, and, most important, the organisms growing under normal conditions readily synthesize large quantities of thiamin and riboflavin [West & Wilson (1, 2, 4)]. True, it might be argued that one has here a "washed cell" growth factor, but acceptance of this somewhat awkward extension leads to admission of a great variety of possible kinds of artifactual "treated cell" growth factors, and the term growth factor loses its simple and accepted significance of referring to an essential trace nutrient that cannot be synthesized by the organism in question from ordinary components of the synthetic medium employed.

Steinberg (2) has claimed the existence of an essential growth factor for *Rhizobium* in addition to coenzyme R, but the evidence for this "rhizobiosin" is very evidently inadequate, and it can be interpreted from the data, indeed, to be simply phosphate.

McBurney, Bollen & Williams point out that *R. meliloti* produces important quantities of pantothenic acid, and make the interesting suggestion that this is of marked value to the host plant, whereas, the plant in its role as symbiont produces coenzyme R of marked value to the bacteria. To date no direct role of coenzyme R is indicated in the nitrogen fixation process. This possibility is not to be excluded, however, in view of the fact that the effect of coenzyme R in *Rhizobium* respiration involves the presence of readily available nitrogen [Allison & Hoover (5); Hoover & Allison], as has also been demonstrated by Burk, Winzler & du Vigneaud in its effect on both respiration and fermentation of yeast.

LITERATURE CITED

ALBRECHT, W. A., *Proc. Soil Sci. Soc. Am.,* **2,** 315 (1937); *Trans. 3d Comm. Intern. Soc. Soil Sci.,* **A,** 71 (1939)

ALBRECHT, W. A., AND McCALLA, T. M., *J. Bact.,* **34,** 455 (1937)

ALLISON, F. E., (1), *J. Agr. Research,* **35,** 915 (1927)

ALLISON, F. E., (2), *Fixed Nitrogen* (H. A. Curtis), chap. ii (Chemical Catalog Co., New York, 1932)

ALLISON, F. E., AND HOOVER, S. R., (1) *J. Bact.,* **27,** 561 (1934)

ALLISON, F. E., AND HOOVER, S. R., (2), *Trans. 3d Intern. Congr. Soil Sci.,* **1,** 145 (1935)

ALLISON, F. E., AND HOOVER, S. R., (3), *J. Bact.,* **31,** 94 (1936)

ALLISON, F. E., AND HOOVER, S. R., (4), *Soil Sci.*, 41, 333 (1936)

ALLISON, F. E., AND HOOVER, S. R., (5), *Trans. 3d Comm. Intern. Soc. Soil Sci.*, A, 32 (1939)

ALLISON, F. E., HOOVER, S. R., AND BURK, D., *Science*, 78, 217 (1933)

ALLISON, F. E., LUDWIG, C. A., HOOVER, S. R., AND MINOR, F. W., *Nature*, 144, 711 (1939)

ALLISON, F. E., AND MINOR, F. W., *Soil Sci.*, 46, 473 (1938); *J. Bact.*, 39, 373 (1940); *Proc. 3d Intern. Congr. Microbiol.*, 486 (1940)

ANDERSON, D. A., *Iowa Agr. Expt. Sta. Research Bull.*, No. 158 (1933)

ARNON, D. I., AND STOUT, P. R., *Plant Physiol.*, 14, 599 (1939)

ASO, K., MIGITA, M., AND IHDA, T., *Soil Sci.*, 48, 1 (1939)

BACH, A., IERMOLIEVA, Z., AND STEPANIAN, M., *Compt. rend. acad. sci. (U.R.S.S.)*, 1, 22 (1934)

BAIER, C. R., *Zentr. Bakt. Parasitenk.*, II, 95, 97 (1936)

BASSALIK, K., AND NEUGEBAUER, J., (1), *Acta Soc. Botan. Polon.*, 8, 213 (1931)

BASSALIK, K., AND NEUGEBAUER, J., (2), *Acta Soc. Botan. Polon.*, 10, 481 (1933)

BASSALIK, K., AND NEUGEBAUER, J., (3), *Acta Soc. Botan. Polon.*, 11, 581 (1934)

BERGER, J., JOHNSON, M. J., AND PETERSON, W. H., *J. Bact.*, 36, 521 (1938)

BERNHEIM, F., AND BERNHEIM, M. L. C., *Cold Spring Harbor Symposia Quant. Biol.*, 7, 174 (1939)

BIRCH-HIRSCHFELD, L., *Arch. Mikrobiol.*, 3, 341 (1932)

BJÄLFVE, G., *Kgl. Landtbruks-Akad. Handl. Tid.*, 74, 963 (1935)

BJÄLFVE, G., NILSSON, R., AND BURSTRÖM, D., *Naturwissenschaften*, 26, 840 (1938); *Lantbruks-Högskol. Ann.*, 7, 291 (1939)

BLOM, J., *Zentr. Bakt. Parasitenk.*, II, 84, 60 (1931)

BONAZZI, A., *Proc. 4th Intern. Soil Sci. Conf.*, III-B, No. 8 (1925)

BOND, G., (1), *Ann. Botany*, 50, 559 (1936)

BOND, G., (2), *Nature*, 140, 683 (1937)

BOND, G., (3), *Nature*, 142, 539 (1938)

BOND, G., AND BOYES, J., *Ann. Botany*, n.s. 3, 901 (1939)

BORTELS, H., (1), *Arch. Mikrobiol.*, 1, 333 (1930)

BORTELS, H., (2), *Zentr. Bakt. Parasitenk.*, II, 87, 476 (1933)

BORTELS, H., (3), *Zentr. Bakt. Parasitenk.*, II, 95, 193 (1936)

BORTELS, H., (4), *Arch. Mikrobiol.*, 8, 1 (1937)

BORTELS, H., (5), *Arch. Mikrobiol.*, 8, 13 (1937)

BORTELS, H., (6), *Ber. deut. botan. Ges.*, 56, 153 (1938)

BORTELS, H., (7), *Zentr. Bakt. Parasitenk.*, I, 131, 494 (1938)

BORTELS, H., (8), *Zentr. Bakt. Parasitenk.*, II, 100, 373 (1939)

BORTELS, H., (9), *Zentr. Bakt. Parasitenk.*, II, 102, 129 (1940)

BURK, D., (1), *J. Phys. Chem.*, 34, 1174, 1195 (1930)

BURK, D., (2), *Proc. 2nd Intern. Congr. Soil. Sci.*, III, 6 (1930)

BURK, D., (3), *Ergeb. Enzymforsch.*, 3, 23 (1934).

BURK, D., (4), *Biokhimiya*, 2, 512 (1937)

BURK, D., (5), (Unpublished work)

BURK, D., AND HORNER, C. K., (1), *Naturwissenschaften*, 23, 259 (1935)

BURK, D., AND HORNER, C. K., (2), *Trans. 3d Intern. Congr. Soil. Sci.*, 1, 148 (1935)

BURK, D., AND HORNER, C. K., (3), *Trans. 3d Intern. Congr. Soil Sci.,* **1,** 152 (1935)

BURK, D., AND HORNER, C. K., (4), *Proc. Am. Soc. Soil Sci.,* **1,** 213 (1937)

BURK, D., AND HORNER, C. K., (5), *Soil Sci.,* **41,** 81 (1936)

BURK, D., AND HORNER, C. K., (6), *Proc. 3d Intern. Congr. Microbiol.,* 489 (1940)

BURK, D., HORNER, C. K., AND LINEWEAVER, H., *J. Cellular Comp. Physiol.,* **1,** 435 (1932)

BURK, D., AND LINEWEAVER, H., (1), *J. Bact.,* **19,** 389 (1930)

BURK, D., AND LINEWEAVER, H., (2), *Arch. Mikrobiol.,* **2,** 155 (1931)

BURK, D., LINEWEAVER, H., AND HORNER, C. K., (1), *Soil Sci.,* **33,** 413, 455 (1932)

BURK, D., LINEWEAVER, H., AND HORNER, C. K., (2), *J. Bact.,* **27,** 325 (1934)

BURK, D., WINZLER, R. J., AND VIGNEAUD, V. DU, *J. Biol. Chem.,* xxi (1941)

BURRIS, R. H., AND MILLER, C. E., *Science,* **93,** 114 (1941)

CLARK, D. G., *Cornell Univ. Agr. Expt. Sta. Mem.,* No. 196 (1936)

COOPER, E. A., DAKER, W. D., AND STACEY, M., *Biochem. J.,* **32,** 1752 (1938)

COOPER, E. A., AND PRESTON, J. F., *J. Soc. Chem. Ind.,* **56,** 1–5 T (1937)

DEMIDENKO, T. T., AND TIMOFEEVA, E. F., *Compt. rend. acad. sci. (U.R.S.S.),* **14,** 231 (1937)

DE ROSE, H. R., EISENMENGER, W. S., AND RITCHIE, W. S., *Mass. State Agr. Expt. Sta. Bull.,* No. 347, 18 (1938)

DMITRIEV, K. A., *Chemisation Socialistic Agr. (U.S.S.R.),* **7,** 80 (1938)

ENDRES, G., (1), *Naturwissenschaften,* **22,** 662 (1934)

ENDRES, G., (2), *Ann.,* **512,** 54 (1934)

ENDRES, G., (3), *Ann.,* **518,** 109 (1935)

ENDRES, G., (4), Private communication (1935)

ENDRES, G., AND KAUFMANN, L., *Ann.,* **535,** 1 (1938)

ENGEL, H., AND ROBERG, M., *Ber. deut. botan. Ges.,* **56,** 337 (1938)

FLIEG, D., *Forschungsdienst,* **6,** 233 (1937)

FRED, E. B., BALDWIN, I. L., AND McCOY, E., "Root Nodule Bacteria and Leguminous Plants," *Univ. Wisconsin Studies Sci.,* No. 5 (1932)

FULLER, J. E., AND RETTGER, L. F., *Soil Sci.,* **31,** 219 (1931)

GEORGI, C. E., AND BEGUIN, A. E., *Nature,* **143,** 25 (1939)

GIÖBEL, G., *New Jersey Agr. Expt. Sta. Bull.,* No. 436 (1926)

GMELIN, *Handbuch Anorg. Chem.,* 29–46 (1934)

GREAVES, J. E., (1), *Soil Sci.,* **6,** 163 (1918)

GREAVES, J. E., (2), *Agricultural Bacteriology,* chaps. 23–25 (Constable & Co., Ltd., London, 1922)

GREAVES, J. E., (3), *Soil Sci.,* **36,** 267 (1933)

GREAVES, J. E., (4), *Proc. 3d Intern. Congr. Microbiol.,* 698 (1940)

GREAVES, J. E., AND ANDERSON, A., *Soil Sci.,* **41,** 197 (1936)

GREAVES, J. E., JONES, L., AND ANDERSON, A., *Soil Sci.,* **49,** 9 (1940)

GREENE, R. A., (1), *Soil Sci.,* **33,** 153 (1932)

GREENE, R. A., (2), *Soil Sci.,* **39,** 327 (1935)

GYÖRGY, P., MELVILLE, D. B., BURK, D., AND VIGNEAUD, V. DU, *Science,* **91,** 243 (1940)

György, P., Rose, C. S., Hofmann, K., Melville, D., and Vigneaud, V. du, *Science, 92,* 609 (1940)

Hamilton, W. B., *J. Bact., 22,* 249 (1931)

Hoover, S. R., and Allison, F. E., *Trans. 3d Intern. Congr. Soil Sci., 1,* 158 (1935)

Hopkins, E. W., Peterson, W. H., and Fred, E. B., *J. Am. Chem. Soc., 52,* 3659 (1930); **53,** 306 (1931)

Horner, G. M., *Univ. Missouri Agr. Expt. Sta. Research Bull.,* No. 232 (1936)

Horner, C. K., and Burk, D., (1), *J. Agr. Research, 48,* 981 (1934)

Horner, C. K., and Burk, D., (2), *Trans. 3d Comm. Intern. Soc. Soil Sci.,* A, 168 (1939)

Horner, C. K., Burk, D., and Allison, F. E. (In press, 1941)

Hutchings, T. B., *Univ. Missouri Agr. Expt. Sta. Research Bull.,* No. 243 (1936)

Isakova, A. A., *Bull. Acad. Sci. U.R.S.S., 9,* 1493 (1933)

Isakova, A. A., and Andrejev, V. A., *Compt. rend. acad. sci. (U.R.S.S.), 18,* 101 (1938)

Jensen, H. L., *Proc. Linnean Soc. N. S. Wales, 65,* 1 (1940)

Kluyver, A. J., and van Reenen, W. J., *Arch. Mikrobiol., 4,* 280 (1933)

Kögl, F., and Tönnis, B., *Z. physiol. Chem., 242,* 43 (1936)

Konishi, K., and Tsuge, T., *J. Agr. Chem. Soc. Japan, 9,* 129 (1933)

Kostyschev, S., Ryskaltschuk, A., and Schwetzova, O., *Z. physiol. Chem., 154,* 1 (1926)

Kostyschev, S., and Scheloumova, A., *Z. physiol. Chem., 198,* 105 (1931)

Kovats, J., *Bull. intern. acad. polon. sci., Classe sci. nat.,* B, I, 91 (1938)

Krzemieniewska, H., *Anz. Akad. Wiss. Krakau,* B, 376 (1910)

Krzemieniewski, S., and Kovats, J., *Bull. intern. acad. polon. sci., Classe sci. math. nat.,* B, I, 169 (1936)

Kubo, H., (1), *Acta Phytochim. (Japan), 10,* 219 (1937)

Kubo, H., (2), *Acta Phytochim. (Japan), 11,* 195 (1939)

Laird, D. G., and West, P. M., *Can. J. Research, 16,* 347 (1938)

Lewis, I. M., *Science, 85,* 16 (1937); *J. Bact., 34,* 191 (1937)

Lineweaver, H., (1), *J. Biol. Chem., 99,* 575 (1933)

Lineweaver, H., (2), *J. Biol. Chem., 122,* 549 (1938)

Lineweaver, H., Burk, D., and Deming, W. E., *J. Am. Chem. Soc., 56,* 225 (1934)

Link, G. K. K., *Nature, 140,* 507 (1937)

Link, G. K. K., and Eggers, V., *Botan. Gaz., 101,* 650 (1940)

Löhnis, M. P., *Soil Sci., 29,* 37 (1930)

Ludwig, C. A., and Allison, F. E., (1), *Botan. Gaz., 98,* 680 (1937)

Ludwig, C. A., and Allison, F. E., (2), *Am. J. Botany, 27,* 719 (1940)

Ludwig, C. A., Allison, F. E., Hoover, S. R., and Minor, F. W., *Botan. Gaz.* (In press, 1941)

Madhok, M. R., *Soil Sci., 49,* 419 (1940)

McBurney, C. H., Bollen, W. B., and Williams, R. J., *Proc. Natl. Acad. Sci. U.S., 21,* 301 (1935)

Meyerhof, O., and Burk, D., *Z. physik. Chem.,* A, **139,** 117 (1928)

Mothes, K., *Forschungsdienst, 5,* 219 (1938)

Mothes, K., and Pietz, J., *Naturwissenschaften, 25,* 201 (1937)

MÜLLER, A., AND STAPP, C., *Arb. biol. Reichs. Land- u. Forstw. Berlin-Dahlem,* **14**, 455 (1925)

NICOL, H., *Monthly Bull. Agr. Sci. Practice,* **6**, 201; **7**, 241 (1936)

NILSSON, R., *Arch. Mikrobiol.,* **7**, 598 (1936)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (1), *Naturwissenschaften,* **26**, 284 (1938)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (2), *Naturwissenschaften,* **26**, 661 (1938)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (3), *Lantbruks-Högskol. Ann.,* **5**, 291 (1938)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (4), *Lantbruks-Högskol. Ann.,* **6**, 299 (1938)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (5), *Lantbruks-Högskol. Ann.,* **7**, 51 (1938)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM., D., (6), *Lantbruks-Högskol. Ann.,* **7**, 301 (1939)

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D., (7), *Naturwissenschaften,* **27**, 389 (1939)

NOWOTNOWNA, A., *J. Agr. Sci.,* **27**, 503 (1937)

OBRATZOVA, A. A., MINENKOV, A. R., REVIAKINA, E. Y., HOLLAND, D. M., AND KRASSILNIKOVA, A. J., *Microbiology,* **6**, 877 (1937)

ORCUTT, F. S., *Soil Sci.,* **44**, 203 (1937)

PIETZ, J., *Zentr. Bakt. Parasitenk., II,* **99**, 1 (1938)

PIRSCHLE, K., *Ergeb. Biol.,* **15**, 67 (1938)

RIPPEL, A., *Arch. Mikrobiol.,* **7**, 210, 590 (1936); **8**, 479 (1937)

ROBERG, M., *Jahrb. Wiss. Botan.,* **82**, 65 (1935)

ROMASHEV, P. I., *Pedology (U.S.S.R.),* No. 4, 99 (1939)

RUBEN, S., HASSID, W. Z., AND KAMEN, M. D., *Science,* **91**, 578 (1940)

SCHOLZ, W., *Bodenkunde u. Pflanzenernähr.,* **15**, 47 (1939)

SCHRÖDER, M., *Zentr. Bakt. Parasitenk., II,* **85**, 177 (1932)

SHAPTER, R. E., *J. Council Sci. Ind. Research,* **12**, 23 (1939)

SIFFERD, R. H., AND ANDERSON, R. J., *Z. physiol. Chem.,* **239**, 270 (1936)

STAPP, C., (1), *Proc. Intern. Congr. Soil Sci.,* **III**, 1 (1927)

STAPP, C., (2), *Proc. 2nd Intern. Congr. Microbiol.,* 146 (1936)

STAPP, C., AND BORTELS, H., *Zentr. Bakt. Parasitenk., II,* **94**, 497 (1936)

STAPP, C., AND RUSCHMANN, G., *Arb. biol. Reichs. Land- u. Forstw. Berlin-Dahlem,* **13**, 305 (1924)

STEINBERG, R. A., (1), *J. Agr. Research,* **52**, 439 (1936)

STEINBERG, R. A., (2), *J. Agr. Research,* **57**, 461 (1938)

STEPHENSON, M., *Bacterial Metabolism,* chap. viii (Longmans, Green and Co., London, 1939)

STRONG, T. H., AND TRUMBLE, H. C., *Nature,* **143**, 286 (1939)

SULLIVAN, J. L., *Iowa State Coll. J. Sci.,* **14**, 87 (1939)

SUOMALAINEN, H., *Suomen Kemistilchti, B.,* **13**, 18 (1940)

THIMANN, K. V., (1), *Proc. Natl. Acad. Sci. U.S.,* **22**, 511 (1936); *Trans. 3d Comm. Intern. Soc. Soil Sci.,* **A**, 24 (1939)

THOMPSON, L. G., *J. Agr. Research,* **45**, 149 (1932)

THORNE, D. W., *Iowa State Coll. J. Sci.,* **11**, 125 (1936)

THORNE, D. W., AND WALKER, R. H., (1), *Proc. Iowa Acad. Sci.*, **41**, 63 (1934)

THORNE, D. W., AND WALKER, R. H., (2), *Proc. Iowa Acad. Sci.*, **42**, 89 (1935)

THORNE, D. W., AND WALKER, R. H., (3), *J. Bact.*, **30**, 33 (1935)

THORNE, D. W., AND WALKER, R. H., (4), *Soil Sci.*, **42**, 231 (1936)

THORNE, D. W., AND WALKER, R. H., (5), *Soil Sci.*, **42**, 301 (1936)

THORNTON, H. G., AND NICOL, H., *J. Agr. Sci.*, **24**, 540 (1934) ; **26**, 173 (1936)

TRUMBLE, H. C., AND STRONG, T. H., *Australia Council Sci. Ind. Research Bull.*, No. 105, 11 (1937)

UMBREIT, W. W., AND BURRIS, R. H., *Soil Sci.*, **45**, 111 (1938)

UMBREIT, W. W., AND WILSON, P. W., *Trans. 3d Comm. Intern. Soc. Soil Sci.*, **A**, 29 (1939)

USPENSKY, E. E., "Soil Microbiology in the U.S.S.R. (1917–1932)," *Trans. 3d Comm. Intern. Soc. Soil Sci.*, **A**, 7 (1933)

VAN NIEL, C. B., *Arch. Mikrobiol.*, **6**, 215 (1935)

VIGNEAUD, V. DU, MELVILLE, D. B., GYÖRGY, P., AND ROSE, C. S., *Science*, **92**, 62 (1940)

VIRTANEN, A. I., (1), *J. Soc. Chem. Ind.*, **54**, 1015 (1935)

VIRTANEN, A. I., (2), *Nature*, **138**, 880 (1936)

VIRTANEN, A. I., (3), *Arch. Mikrobiol.*, **7**, 488 (1936)

VIRTANEN, A. I., (4), *Suomen Kemistilehti*, **9**, 69 (1936)

VIRTANEN, A. I., (5), *Cattle Fodder and Human Nutrition* (Cambridge University Press, London, 1938)

VIRTANEN, A. I., (6), *Trans. 3d Comm. Intern. Soc. Soil Sci.*, **A**, 4 (1939)

VIRTANEN, A. I., AND ARHIMO, A. A., (1), *Suomen Kemistilehti*, *B*, **12**, 24 (1939)

VIRTANEN, A. I., AND ARHIMO, A. A., (2), *Nature*, **144**, 36 (1939)

VIRTANEN, A. I., AND ARHIMO, A. A., (3), *Trans. 3d Comm. Intern. Soc. Soil Sci.*, **B**, 20 (1939)

VIRTANEN, A. I., AND HAUSEN, S. V., *J. Agr. Sci.*, **25**, 278, 290 (1935) ; **26**, 281 (1936)

VIRTANEN, A. I., HAUSEN, S. V., AND LAINE, T., *J. Agr. Sci.*, **27**, 332, 584 (1937)

VIRTANEN, A. I., AND LAINE, T., (1), *Suomen Kemistilehti*, *B*, **9**, 12 (1936)

VIRTANEN, A. I., AND LAINE, T., (2), *Biochem. J.*, **30**, 377 (1936)

VIRTANEN, A. I., AND LAINE, T., (3), *Suomen Kemistilehti*, *B*, **10**, 2 (1937)

VIRTANEN, A. I., AND LAINE, T., (4), *Suomen Kemistilehti*, *B*, **10**, 24 (1937)

VIRTANEN, A. I., AND LAINE, T., (5), *Enzymologia*, **3**, 266 (1937)

VIRTANEN, A. I., AND LAINE, T., (6), *Nature*, **142**, 165 (1938)

VIRTANEN, A. I., AND LAINE, T., (7), *Nature*, **141**, 748 (1938)

VIRTANEN, A. I., AND LAINE, T., (8), *Biochem. J.*, **33**, 412 (1939)

VIRTANEN, A. I., LAINE, T., AND HAUSEN, S. V., *Suomen Kemistilehti*, *B*, **9**, 1 (1936)

VIRTANEN, A. I., LAINE, T., AND RINTALA, P., *Enzymologia*, **9**, 53 (1940)

VIRTANEN, A. I., SAASTAMOINEN, S., AND LAINE, T., *Suomen Kemistilehti*, *B*, **9**, 28 (1937)

VIRTANEN, A. I., AND TORNIAINEN, M., (1), *Suomen Kemistilehti*, *B*, **9**, 13 (1936)

VIRTANEN, A. I., AND TORNIAINEN, M., (2), *Nature*, **145**, 25 (1940)

WEST, P. M., AND WILSON, P. W., (1), *Science*, **88**, 334 (1938)

WEST, P. M., AND WILSON, P. W., (2), *Nature,* **142,** 397 (1938)

WEST, P. M., AND WILSON, P. W., (3), *Science,* **89,** 607 (1939)

WEST, P. M., AND WILSON, P. W., (4), *J. Bact.,* **37,** 161 (1939)

WEST, P. M., AND WILSON, P. W., (5), *Enzymologia,* **8,** 152 (1940)

WILSON, J., AND WILSON, P. W., *J. Bact.,* **41,** 84 (1941)

WILSON, J. K., AND WILSON, B. D., *Cornell Univ. Agr. Expt. Sta. Mem.* No. 148 (1933)

WILSON, P. W., (1), *J. Am. Chem. Soc.,* **58,** 1256 (1936)

WILSON, P. W., (2), *Nature,* **140,** 154 (1937)

WILSON, P. W., (3), *Botan. Rev.,* **3,** 365 (1937)

WILSON, P. W., (4), *Ergeb. Enzymforsch.,* **8,** 13 (1939)

WILSON, P. W., (5), *The Biochemistry of Symbiotic Nitrogen Fixation* (University of Wisconsin Press, Madison, 1940)

WILSON, P. W., AND BURTON, J. C., *J. Agr. Sci.,* **28,** 307 (1938)

WILSON, P. W., AND FRED, E. B., *Proc. Natl. Acad. Sci. U.S.,* **23,** 503 (1937)

WILSON, P. W., AND SARLES, W. B., *Tabulae Biol.,* **17,** 338 (1939)

WILSON, P. W., AND UMBREIT, W. W., (1), *Zentr. Bakt. Parasitenk., II,* **96,** 402 (1937)

WILSON, P. W., AND UMBREIT, W. W., (2), *Arch. Mikrobiol.,* **8,** 440 (1937)

WILSON, P. W., UMBREIT, W. W., AND LEE, S. B., *Biochem. J.,* **32,** 2084 (1938)

WILSON, P. W., AND WYSS, O., (1), *Proc. Soil Sci. Soc. Am.,* **2,** 289 (1937)

WILSON, P. W., AND WYSS, O., (2), *Trans. 3d Comm. Intern. Soc. Soil Sci.,* **B,** 14 (1939)

WINOGRADSKY, S., (1), *Compt. rend.,* **190,** 661 (1930)

WINOGRADSKY, S., (2), *Ann. inst. Pasteur,* **48,** 269 (1932)

WINOGRADSKY, S., (3), *Compt. rend.,* **197,** 209 (1933)

WINOGRADSKY, S., (4), *Soil Sci.,* **40,** 59 (1935)

WINOGRADSKY, S., (5), *Zentr. Bakt. Parasitenk., II,* **97,** 399 (1938)

WINOGRADSKY, S., (6), *Trans. 3d Comm. Intern. Soc. Soil Sci.,* **B,** 37 (1939)

WINOGRADSKY, S., (7), *Compt. rend.,* **209,** 616 (1939)

WINOGRADSKY, S., AND WINOGRADSKY, H., *Ann. inst. Pasteur,* **56,** 221 (1936)

WYSS, O., BURRIS, R. H., AND WILSON, P. W., *Proc. Soc. Exptl. Biol. Med.,* **40,** 372 (1939)

WYSS, O., AND WILSON, P. W., *J. Bact.,* **41,** 186 (1941)

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PROPERTIES OF PROTEIN MONOLAYERS

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A substance will form a monomolecular film on water if its molecules contain a soluble and an insoluble group. Spreading is impossible when the molecule as a whole is soluble, because it contains too many polar groups (e.g., glucose), and spreading does not occur when the molecule is insoluble, because it is built up from nonpolar groups only (e.g., paraffin). This simple concept gives the rudiments of the principles concerned in spreading; it suffices to give an idea of the differences in spreading tendencies of different substances.

In order to indicate the significance of spreading in biology, it might be sufficient to enumerate all the substances present in living organisms that are capable of spreading. Although such an enumeration would include the most important materials in animals and plants, it would be inadequate to depict the importance of spreading for biological systems. This may be illustrated by the observation that very active substances, although not capillary-active and not capable of spreading themselves, nevertheless are kept at a surface by being linked to other molecules, chiefly proteins. The protein acts as a "carrier"—it is more adequate to say that it acts as a boat—because it fixes the active "prosthetic" group to a water surface.

Among the building stones of animal and plant organisms, very few are so soluble as to be nonspreading. To this group belong glucose and other sugars, urea, and inorganic substances. But it would be erroneous to conclude that those substances are not involved in the phenomenon of spreading, because most of them are either present at the surface by being fixed to ionized or nonionized groups in other spreading molecules (lipids, proteins) or are active in promoting the spreading tendency of the spreading substances.

The biologist, therefore, has to interest himself in the study of spreading phenomena because a large proportion of the building stones of animal and plant organisms are spreading substances, and it is necessary to understand the spreading properties of these materials. But it is easy to see that a modern biologist has also other reasons for interest in the study of spreading phenomena. In recent years many facts of importance have been observed relative to the

properties of monomolecular layers. Investigations have dealt with surface areas, viscosity, elasticity and compressibility, potential differences, and optical and x-ray measurements. It is clear that whoever studies one of these properties of living matter has to take account of the properties of monolayers of proteins and lipids. This is perhaps best understood by the example of the potential differences existing above a monolayer spread on water. A layer of protein having a thickness of 10 Å produces a potential difference between the water and the upper surface of the protein of the order of 200 to 300 mv. This is a value familiar to those working with bioelectric phenomena. General reviews have been written by Adam (1), Gorter (2), Langmuir (3, 4, 5) and Neurath & Bull (6).

The areas of protein monolayers.—Study of the area of monolayers of different substances has shown that as a rule the molecules have the same form and dimensions as indicated by structural formulas and as derived from x-ray or electron diffraction measurements. The spreading area of fatty acids is independent, within certain limits, of the length of the chain. Films of long-chain molecules are rather thick, and in agreement with this, the surface charge and potential differences above these films are high. Fats and lipids like lecithin and cephalin form a monolayer with a thickness equal to the square root of the surface area, permitting the conclusion that the molecules are more or less spherical. Proteins behave very unexpectedly. If they spread completely, they occupy a surface corresponding to a film thickness of 10 Å, and a "standard value" of 1 sq. m. per mg. This value is much lower than was foreseen, because several proteins (mol. wt. *ca.* 35,000) (7) have spherical molecules in solution (ovalbumin, myoglobin, insulin), the diameter of a sphere being 45 Å. Now some proteins (mol. wt. *ca.* 210,000) have a much larger diameter in solution, but nevertheless form films of the same thickness, 10 Å. This discrepancy is not due to an error in measurement, because the value of 1 sq. m. per mg. is confirmed by many experiments (see Table I).

Moreover, Astbury *et al.* (25) have measured directly the thickness of multilayers of ovalbumin, prepared in this laboratory on metal slides by the technique of Blodgett, and have found the thickness of one layer to be 10 Å. The only possible explanation for the thinness of a spread protein layer is that its form at a water surface is not spherical but a flat platelet. This platelet is formed from the sphere in solution by the unfolding of the molecule. This is also in agreement

with the thickness of a layer of a tripeptide (24) of α-aminocaprylic acid (1.2 sq. m. per mg.), the larger factor being due to the greater length of each side chain (C_8). The polypeptide chains lie flat on the surface with most of their polar groups in contact with the water. It is perhaps advisable to study separately the different factors which produce or promote this unfolding (22), and the influence of these factors on the spread layer of 10 Å thickness which is already formed.

TABLE I

AREAS OF PROTEIN MONOLAYERS

Protein	Solvent	Solution in the tray	Area,* sq. m. per mg.	Reference
Pseudoglobulin	$(NH_4)_2SO_4$ sol.	0.1 N HCl, pH 1	1.0	(8)
		0.0033 M CH_3COONa buffer, pH 5.2†	0.96	(9)
Insulin	HCl, pH 2.5	0.1 N HCl, pH 1	1.0	(10)
	HCl, pH 2.0	0.1 N HCl, pH 1–1.5	1.0	(11)
Urease	H_2O	0.1 N HCl, pH 1	1.02	(12)
Fibrinogen	H_2O, containing trypsin, 1/500	0.1 N HCl, pH 1	1.0	(13)
	H_2O, containing prothrombase, 1/100	0.1 N HCl, pH 1	1.0	
Zein	70% alcohol	0.1 N HCl, pH 1	1.08	(11)
		0.0033 M CH_3COONa buffer, pH 5.4†	1.06	
9 different proteins‡		0.1 N HCl, pH 1	ca. 1.0	(14)
Pepsin	0.1 N HCl + 10% glycerol	0.1 N HCl, pH 1	1.0–1.04	(15)
		dilute HCl, pH 2.7†	1.0	
Trypsin	0.001 N HCl + $(NH_4)_2SO_4$	0.1 N HCl, pH 1	1.0	(15)
		0.0033 M HCl + Na_2CO_3, pH 6.6†	1.0	

* Area found by linear extrapolation of pressure-area curves to zero pressure.
† Approximately isoelectric.
‡ Egg albumin; Bence-Jones protein; hemoglobin; serum albumin; serum globulin; r-phycoerythrin; r-phycocyan; c-phycocyan; amandin.

TABLE I—*Concluded*

Protein	Solvent	Solution in the tray	Area,* sq. m. per mg.	Reference
Myosin	1.2 M KCl, containing trypsin, 1/3200	0.08 M phosphate buffer, pH 7.3–7.4	0.9	(16)
Ovalbumin	H_2O	0.1 N HCl, pH 1	1.02	(17)
		0.0033 M CH$_3$COONa buffer, pH 4.7†	1.0	
		0.1 N HCl, pH 1	0.95	(18)
		0.1 N HCl, pH 1	1.06	(19)
		0.0033 M CH$_3$COONa buffer, pH 4.3	1.06	
		0.0033 M CH$_3$COONa buffer, pH 5.3	1.06	
Ovalbumin, native	H_2O	0.150 M acetate buffer, pH 4.9	1.04	(20)
Ovalbumin, heat-denatured	HCl, pH 2.4			
Serum albumin	0.15 M NaCl	0.15 M NaCl	0.98	(21)
	propyl alcohol + 1.7 M CH$_3$COONa	+ 0.0067 M phosphate, pH 7	0.99	
Hemoglobin				
Muscle hemoglobin, reduced	H_2O	0.0033 M phosphate buffer, pH 6.8†	0.94	(22)
Globin, horse and cow				
Cytochrome-c	1% NaCl + 3% alcohol	0.0033 M buffer, pH 9.6†	1.0	(23)
		conc. buffer, pH 13.0	1.2	
		0.01 M NaHCO$_3$ + 0.002 M Na$_2$CO$_3$, pH 9.4	1.04	
		0.01 M K$_2$CO$_3$, pH 10.0	1.17	
Tripeptide		0.0033 M buffer, pH 3.5	1.2	(24)

Factors influencing size and properties of protein monolayers.—
If one studies the influence of time, pH, electrolyte concentration,
valency of the ions, alkaline substances, and di- and polyvalent acids
on the spreading or unfolding of a protein, one finds that the curve
representing the variation of area with pH has a W form. Minima
are observed on both sides of the isoelectric point of the protein,
and complete spreading occurs when the solutions are either at the
isoelectric point or have pH values of 1 or 13. These curves have
recently been confirmed by Seastone (26). Now this holds good only
if the film is studied a short time (1 min.) after the protein solution
has been blown on the surface at room temperature and in the absence
of strong concentrations of monovalent ions or of bivalent and triva-
lent ions in smaller amounts. Time has the tendency to increase the
area near the isoelectric point but has no influence at the maxima
and at the lowest minimum. The explanation of these facts is that
all the factors mentioned act on the unfolding of the protein molecule
by increasing the probability of contact of the polar groups that are
covering the surface of the sphere of the molecules in solution, with
the surface of the water in the tray. Minima are due not to a ten-fold
greater thickness of a beautifully spread layer, but to an incomplete
formation of the monolayer. The protein not only does not unfold
but also goes partly into solution. That this explanation is true follows
from the inhomogeneity observed by optical (1, 27) and electrical
(19) measurements of the water surface at these minima and from
the examination of the underlying water in the tray, which may con-
tain dissolved protein at these minima.

Moreover, Langmuir (3, 4, 5) has shown that the thickness of a
protein layer, formed under conditions which give minima in the
pressure-area curve, after being transferred to a metal plate, is equal
to the thickness of a well-spread layer. Other facts are also in agree-
ment with the above explanation. Among these is the very interesting
observation that spreading of a given protein under unfavorable con-
ditions is promoted on the acid side of the isoelectric point not only
by the addition of polyvalent acids to the water in the tray, but also
by coupling the acid to the protein and then spreading it on water
without further addition of free acid. We have cited in Schmidt's
handbook [(2), p. 441] examples of the spreading of protein com-
plexes, in particular the complexes of ovalbumin with tartrazine and
of pepsin with spermidine. In these examples, the amino groups of
ovalbumin are bound by the acid sulfite groups of tartrazine, and the

carboxyl groups of pepsin by the amino groups of spermidine. It is obvious that the spreading obtained is not to be considered as a spreading of the protein itself, but rather of the complex formed, in which acid or alkaline groups of the protein molecule hold alkaline or acid groups of the added base or acid.

This led to several experiments, in which different substances (ethyl alcohol, propyl alcohol, hydrochloric acid at pH 2.4, and sodium acetate in 1.7 M concentration) were added to protein in solution, and were found to promote spreading when added to the water in the tray, at a pH which otherwise would not produce spreading. This was in accord with the observations already cited (e.g., tartrazine). Studies have been made on denatured serum albumin and sodium acetate and propyl alcohol (28), denatured ovalbumin and hydrochloric acid (20), alcohol and fibrinogen (29), and alcohol and nerve protein (30).

It is not certain, therefore, that these authors have really studied the spreading of the free protein, and the reviewer is inclined to believe that they have studied the spreading of alcohol-protein complexes.

Insolubility and denaturation of protein monolayers.—It is worth while to consider here two important series of experiments: one series tending to show that a protein that spreads at the surface is rendered insoluble; and the other demonstrating that a protein when spread at a surface is denatured.

A priori it seems necessary to admit that a spread-out layer of protein is neither soluble nor insoluble, the polar groups being soluble in the water, whereas the side chains are not. Experiments seem to indicate, however, that protein monolayers are very insoluble. Langmuir (31, 32) has written:

If the protein films were in thermodynamic equilibrium with an underlying protein solution, the solubility c should increase with [the compressing force] F in accord with Gibbs' equation

$$dF/d \ln c = \sigma kT,$$

where σ is a measure of the amount of protein per unit area in the film in terms of the molecular units, which exist *in solution.*

Assuming a molecular weight of 35,000 for the protein in the solution, a film having a specific area of 1 m.^{2}/mg. corresponds to $\sigma = 1.7 \times 10^{12}$ molecules/sq. cm. (regardless of any assumption regarding the molecular weight of the protein in the monolayer). Thus at 20° there should be a 3×10^{14}-fold increase in solubility for each increment of 1 dyne/cm. in the value of F

Since even at the highest compression these protein monolayers have no measurable solubility, we must conclude that the spreading of a monolayer of protein from solution is a process involving irreversibility to an extraordinary degree.

It is generally accepted that by compressing the film very greatly, threads of protein lying parallel to the moving barrier are formed. These threads are very insoluble. Also, the foam produced by shaking a protein solution vigorously (33, 34) is almost insoluble (31, 32). In these two instances a refolding of the molecule has taken place by bringing soluble parts in contact with each other (8, 35). The spread monolayers are intermediate between such threads and membranes, and proteins in solution. The spread monolayers of the protein molecule are transformed into an insoluble form by compression. It is interesting to note that proteins which have been denatured do not spread in the ordinary way. There are several methods, however, of restoring the spreading capacity of a denatured protein solution. One of these (13, 16) consists in adding a trace of a proteolytic enzyme to the denatured protein, e.g., heat-denatured ovalbumin. Another method (21) employs solution of proteins in an aqueous solution of propyl alcohol containing sodium acetate. The simplest method is to add sufficient hydrochloric acid to a solution of denatured protein to obtain a pH of 2.4. This restores the spreading capacity completely. Bull (20) also studied the heat denaturation and urea denaturation of ovalbumin. The spreading areas (see Table I) and the compressibilities were found to be the same, within the probable error, for native and heat-denatured protein: native protein, 0.0157 ± 0.0002 cm. per dyne; heat-denatured protein, 0.0160 ± 0.000206 cm. per dyne.

Activity of enzymes.—Is it possible now to determine whether the protein in a surface monolayer is denatured? Gorter (8, 36) has shown that pepsin and trypsin monolayers, lifted from the surface of water on a wet silk fabric and transferred to a buffer solution of a different pH, one which promotes solution of the enzyme, retain 80 per cent of their original peptizing activity, as demonstrated by their action on casein solutions. This observation indicates that the monolayer is active or can be reconverted to a soluble form possessing full activity. This experiment has been repeated by Langmuir (37), who made use of a rectangular wire frame which was dipped into a tray of water covered by a pepsin monolayer at $F = 9$. On lifting the frame out of the water a film was obtained which had 10 sq. cm. of a

pepsin monolayer on it. In this case also the specific activity, as shown by the clotting of milk, was about 80 per cent of the original activity of the pepsin.

Langmuir has proved that a monolayer of an active protein deposited on plates usually has some chemical activity. It was impossible to prove this for pepsin, because the milk used to test the activity of the pepsin contained substances which rapidly displaced the adsorbed pepsin monolayers from the plate.

Monolayers of urease deposited on plates remained fixed to the plate with unchanged thickness after having been in contact with a solution of urea. This is true only when the hydrophobic groups are present in the surface. Urease, a protein, remained active in a monolayer, as evidenced by the production of alkali in the urea solution. The activity however was only 0.02 of the activity of the same amount of urease when dissolved. The lower activity may have been due to a loss of activity of the urease solution, during the three and a half months it was kept before it was used.

The same type of experiment was performed with catalase by Langmuir & Schaefer (38), who observed some activity which they attributed to unspread molecules. They questioned whether any enzymic activity would be shown by a completely spread protein. They also noted a removal of the catalase from areas of the plate on which bubbles were allowed to form. In their last publication Langmuir & Schaefer (5) concluded:

Further work is planned whether in the case of catalase as with urease the activity of the monolayer is possibly due to unspread molecules enmeshed in the fabric of the spread monolayer.

Harkins, Fourt & Fourt (39) have also studied the activity of catalase in a monolayer. They did not observe the removal of the catalase by the gas bubbles and ascribed this to the use of $0.008\ M$ instead of 3 per cent ($0.9\ M$) hydrogen peroxide. But they have noted losses of thickness of the layer of adsorbed enzyme which were uniform over the whole plate. Catalase was scarcely liberated if a layer of catalase was covered by an outer layer of anticatalase. This double layer was hardly less active than exposed catalase.[1]

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[1] The same holds good for precipitates consisting of catalase and anticatalase (40).

The activity per gm. of adsorbed catalase is only a fifth to a tenth of that in solution. This can only be regarded as a tentative value, for two reasons: (1) The *Kat. f* [i.e., enzyme activity] is lower than for some solutions of crystalline catalase, which indicates the presence of denatured protein or an impurity which might be preferentially reducing the activity on the plates. (2) The effect of drying and aging may have impaired the activity, independent of the effects of adsorption alone.

The first hypothesis has been used by Langmuir & Schaefer (37) to explain the fact that a commercial pepsin, when adsorbed on the plate, was more active than a pure preparation. Here a preferential adsorption of the active enzyme was assumed.

We must conclude that it has not yet been proved that monolayers of enzymes adsorbed on a plate are active when they are not liberated into the solution. In any case they can regain their activity after having been spread, and if they are denatured, the denaturation is reversible. This fact is of considerable importance for the biologist, because if proven, it would enable him to conceive a model of a surface in a cell on which the enzyme is deposited and where it shows its activity.

A very large and rapidly increasing group of enzymes and other active substances has been shown to be built up of protein. From some enzymes an active prosthetic group can be reversibly dissociated from the protein; in other enzymes this splitting has not been achieved. The following are some examples of active substances which have been studied by the spreading method: hemoglobin (22), cytochrome-*c* (22, 23), catalase (38, 39), hemocyanin (26), insulin (5, 10, 11), pepsin (15, 37), trypsin (15), and urease (41, 42, 43). Many other complex proteins remain for further study.[2]

Differences of the protein spreading through fixation to prosthetic groups are probable [see (2), p. 441]. Some of the prosthetic groups are themselves capable of spreading on a water surface, while others (e.g., thyroxin) are not. In any case the character of the spreading phenomenon is modified by the fixation to proteins, *v.i.*

Surface pressure of protein monolayers.—During recent years many contributions have been made to the study of the properties of monolayers. Many new methods have been advocated and most of them have served to give more insight into the structure and properties of proteins. Several attempts to study more accurately the

[2] *Ann. Rev. Biochem.*, 7, 37 (1938); 8, 1–80 (1939).

surface pressure of monolayers (44 to 48) have yielded few new aspects. One new fact may be mentioned: the influence of traces of ions such as calcium and copper on the size and properties of monolayers of stearic acid.

Elasticity and compressibility.—Bateman & Chambers (49) have obtained data for the elasticity of egg albumin films, which give no further insight than the data on compressibility heretofore in use. They use the formula

$$M_s = -A \, \frac{\mathrm{d}F}{\mathrm{d}A} \, ,$$

and draw curves of the factor M_s against F. [F is force in dynes per cm., M_s is surface elasticity in dynes per cm., and A is area in apparent sq. m. per mg.—EDITOR.]

Neurath & Bull (6) give the formula for the calculation of a coefficient of compressibility:

$$-\frac{1}{A_0}\left(\frac{\mathrm{d}A}{\mathrm{d}F}\right) .$$

A_0 is the film area found by extrapolating the linear portion of the pressure-area curve to zero pressure and $\mathrm{d}A/\mathrm{d}F$ is the reciprocal of the slope of the linear portion of the pressure-area curve.

They found like Bateman & Chambers (49) a distinct minimum in the coefficient of compressibility at 14 dynes.

TABLE II

COMPRESSIBILITIES OF PROTEIN FILMS SPREAD ON ISOELECTRIC
BUFFER SOLUTIONS

Protein	Coefficient of compressibility in centimeters per dyne	Calculated from the data of
Egg albumin	0.0160	Philippi (19), Bull (20)
Egg albumin	0.0145	Neurath (50)
Serum albumin	0.0242	Philippi (19)
Serum albumin	0.0230	Neurath (50)
Lactoglobulin	0.0187	Philippi (19)
Gliadin	About 0.028	Hughes & Rideal (51)
Cytochrome-c	0.029	Harkins & Anderson (23)
Palmitic acid	0.0021	Adam (1)

We quote here the values for compressibilities as given by Neurath & Bull (6).

Many proteins show a high compressibility which is completely reversible even after a compression to one fourth of the area. Others do not show the complete reversibility. Exactly what happens when a monolayer of protein is compressed, is not known. It is very probable that the polypeptide chains after compression lie roughly parallel to the glass barrier (25), whereas the side chains of the different amino acids lie under the surface or stand up above the surface. As the compression is increased, the tilt of these side chains approaches 90°. Increase of the hydrophobic character when compressing from 0.5 to 1.5 dynes has been observed (20). Ultimately the chains are forced out of the water [see, however, Bull (20)] and this leads to the formation of very thin fibers, lying once more parallel to the glass barrier or to the floating strip of the balance.

What happens may be illustrated by the model of Devaux & Pallu (52), which consists of a layer of grain seeds lying on a clean surface of mercury. When the surface is compressed with a glass barrier, the seeds are lifted so that they occupy less space and finally jump out of the surface. This corresponds to the breaking of the monolayer.

Viscosity of monolayers.—One of the interesting facts that can be observed in the compression of protein monolayers is a sudden increase in viscosity at a certain pressure, as is indicated in Table III [Langmuir & Schaefer (5)].

TABLE III

ABSOLUTE VISCOSITIES OF PROTEIN MONOLAYERS

Protein	M_s in grams per second			
	$F = 2$ dynes	$F = 6$ dynes	$F = 10$ dynes	$F = 16.5$ dynes
Hemoglobin	0.024	0.12	0.37	...
Insulin	0.004	0.028	0.10	...
Pepsin	0.68	0.75	1.5	150
Trypsin	0.009	0.23	0.97	...

$M^* = 131.8$; $D^* = 4.5$; pH $= 5.8$; $T = 25°$ C.; $t =$ about 34 sec.

* [These are constants of the apparatus used. Cf. (60).—EDITOR.]

When the viscosity is high we are dealing with the "gel" state as predicted by Hughes & Rideal (51). The compression of a monolayer

of protein also produces an increase in the potential difference. This is caused by the greater number of charged ions per sq. cm. and by a greater thickness of the layer corresponding to a diminishing tilt of the molecule.

Most methods for determining the viscosity of monolayers are new. Some of these make use of a slit viscosimeter (53 to 57). The monolayer is pressed through a narrow slit and the velocity is measured as a function of the pressure. Other methods use a vibrating vane or a circular disc, suspended on a torsion wire, and determine the damping which is produced by a monolayer (58, 59, 60). Both methods are very useful and give very large differences between the viscosities of different kinds of monolayers of proteins, some having viscosities a thousand times larger than others (5). See Table III.

Multilayers.—In a great number of experiments use has been made of the method of Blodgett & Langmuir (62) to build up films on glass slides, metal plates, or ebonite plates. Although these multilayers show many interesting properties, it has not always been easy to derive from their study the properties and structure of the monolayers. For the study of multilayers optical methods have been used, mostly to determine the thickness of a calculated number of monolayers (62, 63, 64, 65). Also electrical properties have been employed by many experimenters. Here the difficulties have not been completely surmounted, and there is still no general agreement, whether the high potential differences observed in some kinds of monolayers are due to a surface charge on the outer layer only (66, 67, 68, 69), or to a volume charge, each layer contributing its part to the potential difference (70).[3] Measurements of resistance and capacity and of dielectric constants (71) of deposited layers of calcium stearate have also been made (72).

From the study of these multilayers it has been possible to calculate the thickness of monolayers of proteins. When care is taken that the multilayers are prepared by dipping through a surface which is covered by a completely spread monolayer of proteins, the thickness is 9.5 to 10 Å, a number which agrees very well with the magnitude of the spread area of 1 sq. m. per mg.[4] X-ray determinations also

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[3] It is not necessarily true that the potential difference is due to a dipole moment, as has been assumed by most previous authors (1).

[4] In the literature one finds a thickness of 7 Å corresponding with an area of 1 sq. m. per mg. This discrepancy is due to the value assumed for the specific

give the value of 9.5 Å for the distance between two polypeptide chains (73, 74, 75).

Interaction between proteins and other substances on the surface. —Different methods have been used to study the behavior of mixed films. The simplest example is given by a mixture of fatty acids and liquid paraffin (76), in which 0.3 mole of paraffin was added to 1.0 mole of fatty acid. This had a strong effect in increasing the area occupied by the stearic acid, and had the same influence, therefore, as when the spreading was studied at an interface between water and benzene. Alexander & Teorell (77), and Adam, Askew & Pankhurst (78) have observed the same effect when studying the spreading of different acids on solutions of phenol, butyric acid, and butyl alcohol. There was a strong effect observed, as in previous experiments by Rideal and his school who injected the second substance under the spread monolayer. More interesting for the biologist are observations of the spreading of a mixed layer of amines and organic acids dissolved in the underlying solution. Cockbain & Schulman (79) observed different possibilities—penetration of the acids between the heads of the amine molecules, or the ejection of the amines from the surface. The interaction has been measured by the method of surface pressure and surface potentials.

An important attempt to elucidate the interaction of carcinogenic compounds, polycyclic hydrocarbons with cholesterol, in surface films was made by Clowes, Davis & Krahl (80).

The endeavor of Langmuir *et al.* to study phenomena which might be of interest to the biologist should also be mentioned. The interaction of different sterols and digitonin was studied by this method, and also by the use of built-up films formed according to the method of Blodgett (81, 82). The interaction between digitonin and cholesterol has also been studied by Schulman & Stenhagen (83).

The interaction between proteins and other substances is of special importance [cf. (2), p. 441].

Injection of a surface-active substance underneath a surface film of protein affects greatly the potential and surface pressure. A considerable increase of surface pressure can occur if the injected mole-

thickness of a monolayer, 7.5 Å corresponding to a specific volume of 0.75; 10 Å corresponds to a specific volume of 1.0. This latter value seems more probable.

cules penetrate the film. This has been observed with mixed films of gliadin and cholesterol (84) and of gliadin and tripalmitin. Penetration can be prevented by increasing the pressure of the protein monolayer. Mixed films of gliadin and triolein are liquid. Displacement of egg albumin molecules in a monolayer occurs if long-chain fatty acids are injected beneath the protein film under high pressure.

Neurath (85) has shown that mixed films of a protein and a fatty acid can be formed by spreading these substances from solution. Only with films containing a few molecules of myristic acid per molecule of egg albumin was an increase of the area per fatty acid molecule observed, assuming that the size of the protein molecule was not modified. Spreading occurred on the surface of $0.1 N$ HCl. Neurath explained the facts by assuming that the films of fatty acid were in the vapor-expanded or gaseous state because of a sharp decrease of the lateral adhesion between the molecules of the fatty acid, brought about by attractive forces acting between the carboxyl groups of the fatty acids and the positively charged groups of the protein film. Nevertheless the mixed film remained solid.

Membranes of mixed monolayers.—As the ultimate goal of these experiments, many observers have in mind the construction of models comparable to structures in living cells. It is generally accepted that cell walls sometimes consist of proteins and lipids. It even appears that the amount of lipids in erythrocytes does not exceed one or two layers. Old experiments of Gorter (86) have recently been confirmed by Dervichian & Macheboeuf (87) and Winkler (88). It is, therefore, of great interest to the biologist that experiments [see also (94)] have already been made to build an artificial membrane from protein and lipids (89). Langmuir & Waugh prepared a 0.5 per cent aqueous solution of egg albumin and covered it with a solution of 0.5 per cent lecithin in benzene and tried to transfer the membranes formed at the interface to a metal plate containing several holes. That this attempt was not very successful is not sufficient evidence to induce us to abandon hope in this type of experiment, for after all, the protein in a cell wall is of quite a different nature from ovalbumin, and the lipids of the cell walls are not only lecithin but also cephalin, sphingomyelin, and cholesterol (90).

Also in other respects application of the observed phenomena on monolayers of proteins and lipids to biological problems has been considered. Moyer (91) has given details of experiments on the cataphoresis of fat droplets of milk and concludes that

These data appear to be most closely in accordance with the evidence of L. S. Palmer for the existence of a complex of phospholipids and a "membrane" protein, different from other known milk proteins, composing the fat droplet surface.

A few years ago van Dam calculated that there is just sufficient lecithin present to cover the surface of the fat droplet with a monolayer.

We may also mention here the chlorophyll-protein complex studied by Hanson (95) in the plastids of plants. Janssen (92) has made use of the facts known concerning protein monolayers to build a theory of protein structure and protein synthesis.

A very good survey of all these problems is given in a general discussion on membranes (93) and by Harvey & Danielli (94) in their survey on the properties of the cell surface.

LITERATURE CITED

1. ADAM, N. K., *The Physics and Chemistry of Surfaces,* 2nd ed. (Clarendon Press, Oxford, 1938)
2. GORTER, E., "Surface tension and films" in *The Chemistry of the Amino Acids and Proteins* (C. L. A. SCHMIDT, EDITOR) (Charles C. Thomas, Springfield, Illinois, 1938)
3. LANGMUIR, I., *Proc. Roy Inst. Gt. Brit.,* **30**, 483 (1938)
4. LANGMUIR, I., *Proc. Roy. Soc. (London), A,* **170**, 1–39 (1939)
5. LANGMUIR, I., AND SCHAEFER, V. J., *Chem. Revs.,* **24**, 181–202 (1939)
6. NEURATH, H., AND BULL, H. B., *Chem. Revs.,* **23**, 391–435 (1938)
7. SVEDBERG, T., *Proc. Roy. Soc. (London), B,* **127**, 1–79 (1939)
8. GORTER, E., *Trans. Faraday Soc.,* **33**, 1125–29 (1937)
9. HOOFT, C., *J. physiol. path. gén.,* **36**, 652–68 (1938)
10. GORTER, E., AND MAASKANT, L., *Proc. Acad. Sci. Amsterdam,* **40**, 71–73 (1937)
11. GORTER, E., AND VAN ORMONDT, J., *Proc. Acad. Sci. Amsterdam,* **36**, 922–25 (1933)
12. GORTER, E., AND MAASKANT, L., *Proc. Acad. Sci. Amsterdam,* **40**, 73–75 (1937)
13. GORTER, E., MAASKANT, L., AND VAN LOOKEREN CAMPAGNE, G. J., *Proc. Acad. Sci. Amsterdam,* **39**, 1187–91 (1936)
14. GORTER, E., AND GRENDEL, F., *Proc. Acad. Sci. Amsterdam,* **32**, 770–71 (1929)
15. GORTER, E., *J. Gen. Physiol.,* **18**, 421–26 (1935)
16. GORTER, E., AND VAN ORMONDT, J., *Biochem. J.,* **29**, 48–52 (1935)
17. GORTER, E., AND PHILIPPI, G. T., *Proc. Acad. Sci. Amsterdam,* **37**, 788–93 (1934)
18. GORTER, E., VAN ORMONDT, J., AND DOM, F. J. P., *Proc. Acad. Sci. Amsterdam,* **35**, 838–43 (1932)
19. PHILIPPI, G. T., *Thesis* (Leyden, 1936)
20. BULL, H. B., *J. Biol. Chem.,* **125**, 585–94 (1938)
21. STÄLLBERG, S., AND TEORELL, T., *Trans. Faraday Soc.,* **35**, 1413–16 (1939)
22. JONXIS, J. H. P., *Biochem. J.,* **33**, 1743–51 (1939)
23. HARKINS, W. D., AND ANDERSON, T. F., *J. Biol. Chem.,* **125**, 369–76 (1938)
24. GORTER, E., MEYER, T. M., AND PHILIPPI, G. T., *Proc. Acad. Sci. Amsterdam,* **37**, 355–57 (1934)
25. ASTBURY, W. T., BELL, F. O., GORTER, E., AND VAN ORMONDT, J., *Nature,* **142**, 33–36 (1938)
26. SEASTONE, C. V., *J. Gen. Physiol.,* **21**, 621–29 (1938)
27. FOWKES, F. M., MYERS, R. J., AND HARKINS, W. D., *J. Am. Chem. Soc.,* **59**, 593–95 (1937)
28. STÄLLBERG, S., *Trans. Faraday Soc.,* **35**, 1416 (1939)
29. FOURT, L., AND PERLEY, A., *Proc. Soc. Exptl. Biol. Med.,* **33**, 201–3 (1935)
30. FOURT, L., AND SCHMITT, F. O., *J. Phys. Chem.,* **40**, 989–96 (1936)
31. LANGMUIR, I., *J. Am. Chem. Soc.,* **60**, 1351–60 (1938)
32. LANGMUIR, I., *Proc. Phys. Soc. (London),* **51**, 592–612 (1939)
33. BULL, H. B., *J. Biol. Chem.,* **123**, 17–30 (1938)

34. WU, H., AND WANG, C. F., *J. Biol. Chem.,* **123**, 439–42 (1938)
35. GORTER, E., *Proc. Roy. Soc. (London), B,* **127**, 33–34 (1939)
36. GORTER, E., *Proc. Roy. Soc. (London), A,* **155**, 706–8 (1936)
37. LANGMUIR, I., AND SCHAEFER, V. J., *J. Am. Chem. Soc.,* **60**, 1351–60 (1938)
38. LANGMUIR, I., AND SCHAEFER, V. J., *Chem. Rev.,* **24**, 181 (1939)
39. HARKINS, W. D., FOURT, L., AND FOURT, P. C., *J. Biol. Chem.,* **132**, 111–18 (1940)
40. CAMPBELL, D. H., AND FOURT, L., *J. Biol. Chem.,* **129**, 385 (1939)
41. LANGMUIR, I., AND SCHAEFER, V. J., *J. Am. Chem. Soc.,* **59**, 2075 (1937)
42. NICOLAI, M. F. E., AND WEURMAN, P., *Proc. Acad. Sci. Amsterdam,* **41**, 904–8 (1938)
43. HANSON, E. A., *Thesis* (Leyden, 1938)
44. HARKINS, W. D., AND ANDERSON, T. F., *J. Am. Chem. Soc.,* **59**, 2189–97 (1937)
45. NUTTING, G. D., AND HARKINS, W. D., *J. Am. Chem. Soc.,* **61**, 1180 (1939)
46. GUASTALLA, J., *Compt. rend.,* **206**, 993–95 (1938)
47. GUASTALLA, J., *Compt. rend.,* **208**, 973–74 (1939)
48. DOGNON, A., AND ABRIBAT, M., *Compt. rend. soc. biol.,* **130**, 1000–2 (1939)
49. BATEMAN, J. B., AND CHAMBERS, L. A., *J. Chem. Phys.,* **7**, 244–50 (1939)
50. NEURATH, H., *J. Phys. Chem.,* **40**, 361 (1936)
51. HUGHES, A. H., AND RIDEAL, E. K., *Proc. Roy. Soc. (London), A,* **137**, 62 (1932)
52. DEVAUX, H., AND PALLU, L., *J. phys. radium,* **9**, 441–46 (1938)
53. JOLY, M., *J. phys. radium,* **9**, 345 (1938)
54. JOLY, M., *J. chim. phys.,* **36**, 285–95 (1939)
55. JOLY, M., *Kolloid-Z.,* **89**, 26–35 (1939)
56. HARKINS, W. D., AND MYERS, R. J., *J. Chem. Phys.,* **5**, 601–3 (1937)
57. HARKINS, W. D., AND KIRKWOOD, J. G., *J. Chem. Phys.,* **6**, 53 (1938)
58. FOURT, L., *J. Phys. Chem.,* **43**, 887–99 (1939)
59. HARKINS, W. D., AND BOYD, G. E., *J. Chem. Phys.,* **7**, 203–4 (1939)
60. LANGMUIR, I., AND SCHAEFER, V. J., *J. Am. Chem. Soc.,* **59**, 2400 (1937)
61. BLODGETT, K. B., *J. Phys. Chem.,* **41**, 975–84 (1937)
62. BLODGETT, K. B., AND LANGMUIR, I., *Phys. Rev.,* **51**, 964–82 (1937)
63. BULL, H. B., *J. Phys. Chem.,* **41**, 985–86 (1937)
64. LANGMUIR, I., SCHAEFER, V. J., AND WRINCH, D., *Science,* **85**, 76–80 (1937)
65. LANGMUIR, I., *Science,* **87**, 493–500 (1938)
66. PORTER, E. F., AND WYMAN, J., *J. Am. Chem. Soc.,* **60**, 1083–94 (1938)
67. HARKINS, W. D., AND MATTOON, R. W., *Phys. Rev.,* **53**, 911–12 (1938)
68. HARKINS, W. D., AND MATTOON, R. W., *J. Chem. Phys.,* **7**, 186–97 (1939)
69. LANGMUIR, I., *J. Am. Chem. Soc.,* **60**, 1190–94 (1938)
70. GORANSON, R. W., AND ZISMAN, W. A., *J. Chem. Phys.,* **7**, 492–505 (1939)
71. RACE, H. H., AND REYNOLDS, S. I., *J. Am. Chem. Soc.,* **61**, 1425–32 (1939)
72. ZAHL, P. H., HASKINS, C. P., AND GALLAGHER, D. M., *Trans. Faraday Soc.,* **35**, 308–12 (1939)
73. ASTBURY, W. T., AND BELL, F. O., *Cold Spring Harbor Symposia Quant. Biol.,* **6**, 109–21 (1938)
74. BERNAL, J. D., *Proc. Roy. Soc. (London), A,* **170**, 75–78 (1939)
75. CROWFOOT, D. M., *Proc. Roy. Soc. (London), A,* **164**, 580 (1938)

76. HARKINS, W. D., AND MYERS, R. J., *J. Phys. Chem.,* **40**, 959–71 (1936)
77. ALEXANDER, A. E., AND TEORELL, T., *Trans. Faraday Soc.,* **35**, 727–33 (1939)
78. ADAM, N. K., ASKEW, F. A., AND PANKHURST, K. G. A., *Proc. Roy. Soc. (London), A,* **170**, 485–500 (1939)
79. COCKBAIN, E. G., AND SCHULMAN, J. H., *Trans. Faraday Soc.,* **35**, 716–27 (1939)
80. CLOWES, G. H. A., DAVIS, W. W., AND KRAHL, M. E., *Am. J. Cancer,* **36**, 98–109 (1939)
81. LANGMUIR, I., AND SHAEFER, V. J., *J. Am. Chem. Soc.,* **59**, 1751 (1937)
82. LANGMUIR, I., SCHAEFER, V. J., AND SOBOTKA, H., *J. Am. Chem. Soc.,* **59**, 1751 (1937)
83. SCHULMAN, J. H., AND STENHAGEN, E., *Proc. Roy. Soc. (London), B,* **126**, 356–69 (1938)
84. SCHULMAN, J. H., AND RIDEAL, E. K., *Proc. Roy. Soc. (London), B,* **122**, 29 (1937)
85. NEURATH, H., *J. Phys. Chem.,* **42**, 39–46 (1938)
86. GORTER, E., *J. Exptl. Med.,* **41**, 439 (1925)
87. DERVICHIAN, D. G., AND MACHEBOEUF, M. A., *Compt. rend.,* **206**, 1511–14 (1938)
88. WINKLER, K. C., *Thesis* (Leyden, 1938)
89. LANGMUIR, I., AND WAUGH, D. F., *J. Gen. Physiol.,* **21**, 745–55 (1938)
90. ERICKSON, B. N., WILLIAMS, H. H., BERNSTEIN, S. S., AVRIN, I., JONES, R. L., AND MACY, I. G., *J. Biol. Chem.,* **122**, 515 (1938)
91. MOYER, L. S., *J. Biol. Chem.,* **133**, 29 (1940)
92. JANSSEN, L. W., *Protoplasma,* **33**, 410–26 (1939)
93. "Properties and functions of membranes, natural and artificial," *Trans. Faraday Soc.,* **33**, 912–1148 (1937)
94. HARVEY, E. N., AND DANIELLI, J. F., *Biol. Rev. Cambridge Phil. Soc.,* **13**, 319–41 (1938)
95. HANSON, E. A., *Rec. trav. botan. néerland.,* **36**, 180 (1939)

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LEYDEN, HOLLAND

AUTHOR INDEX

637

SUBJECT INDEX

A

Abrine, racemization by ketene, 95
Absorption spectra, *see* Spectrometric studies *and* specific substances
Acetic acid
amino acid formation and, 276
detoxications and, 275–77
formation of, 275
21-Acetoxy-5-pregnene-3-ol-20-one, 288
Acetylcholine, thiamin interaction, 341
Acetylmethylcarbinol, formation by bacteria, 574
Acetylphosphate, pyruvate oxidation and, 156
Acid-base balance
adrenalectomy and, 291
hippuric acid formation and, 273
phospholipids and, 190
Acrodynia
factors involved in, 350
see also Pyridoxin, deficiency of
Addison's disease, treatment of, 299, 300
Adenase, in blood, 237
Adenine
deamination of, bacterial, 567
isolation from nucleic acids, 228
Adenosine
ammonia from, 236, 237
deamination of, bacterial, 567
isolation from nucleic acids, 228
in yeast, 228, 232, 233
Adenosine deaminase, 236
Adenosine diphosphate
activity of, 233
determination of, 235, 236
structure of, 233, 234
in yeast, 232
Adenosine-5-phosphoric acid
synthesis of, enzymatic, 228
synthesis of, partial, 229
in yeast, 228, 232
Adenosine triphosphate
decomposition by blood, 237
determination of, 235, 236
structure of, 234
thiamin phosphorylation, 13, 57
Adenylic acids
acridine salts, constitution of, 226
aerobic phosphorylations and, 155
bacterial growth and, 556
coenzyme activity and, 183
deamination, bacterial, 567
phosphorylase activity and, 153
pyruvate oxidation and, 156
replacement by inosinic acid, 49

Adenylic acids (*cont.*)
triosephosphate oxidation and, 23
Adrenal cortex, 288–303
androgens and, 320
extirpation
effects of, 291–94
life maintenance diet, 290, 291
life maintenance by riboflavin, 301
filtrate factor deficiency and, 351, 362, 365, 366
functions, 300–3
hormones of
assay of, 290
carbohydrate metabolism and, 171
clinical uses, 299, 300
fractionation of, 294, 295
muscular efficiency and, 171, 172
phosphorylations and, 162
separate effects of, 294–99
structure and activity, 289
synergism with anterior pituitary, 170
see also specific substances
human fetal, hormones of, 289
phosphorylation of riboflavin and, 348, 349
pituitary gland and, 170, 299, 302, 303
Adrenal gland
dimethylsulfone in, 127
size of, hormones and, 295, 296, 320
Adrenosterone, structure of, 516
Aetiozymase, 338
Agar, nature of, 85
Alanine, naturally occurring, configuration of, 92
β-Alanine
dioxyacyl derivatives, isolation of, 104
as a growth factor, 502, 557
from pantothenic acid, 358
d-Alanine
optical rotation of, 99
oxidation, enzymatic, inhibition of, 8
dl-Alanine, resolution of, 99
l-Alanine
determination of, 101
optical rotation of, 99
Albumins
egg
denatured, 112
reducing groups of, 110
peptic digestion of, 35
serum, conjugated, preparation of, 110
Alcamines, optically active, 100
Alcaptonuria, ascorbic acid and, 201, 376
Aldobionic acid
conversion to disaccharides, 85

Bromelin, activation of, 37
Bromide
  accumulation by plants, 472, 473
  excretion of, adrenal cortex and, 292
Bromobenzene, metabolism of, 278, 279
2,3-Butylene glycol, formation by bacteria, 573

C

C_4-dicarboxylic acids, catalytic activity of, 154, 155
Calcification, cereals and, 403, 404
Calcium
  absorption
    calciferol and, 403
    by plants, 472
  availability of, 404
  Azotobacter requirement, 608
  in blood of fowls, estrogens and, 311
  deficiency in animals, 454
  dietary deficiency
    incidence of, 436, 443
    income and, 442
  plant growth and, 479–81
    boron and, 479
    magnesium and, 480
    nitrate absorption, 479, 480
  in plants, 454, 455
  in soils, 454
Canavanine, configuration of, 93
Cancer
  d-peptides hydrolysis, 39
  proteolytic enzymes and, 39–41
Capillary permeability
  adrenal cortex and, 291, 292
  ascorbic acid and, 372, 378
  citrin and, 378, 379
Carbohydrases, specificity of, 50
Carbohydrate metabolism, 151–75
  absorption, intestinal, 161–63
    adrenal cortex and, 162, 292
    configuration and, 162
    phosphorylation and, 161, 162
    selective, 161, 162
  fat metabolism and, 173, 174, 184
    thiamin and, 340
  naphthalene poisoning and, 267
  thiamin and, 340, 341
  zinc and, 56
  see also Glycolysis; Pyruvic acid, oxidation, etc.
Carbohydrates, 65–86
  of plants, *see* Plant carbohydrates
  see also specific substances
Carbon, microdetermination of, 103
Carbon dioxide
  bacterial growth and, 17, 560–61, 593
  bacterial metabolism and, 593
  catalytic effect, 561
  in cell respiration, 16–18

Carbon dioxide (*cont.*)
  in cell respiration (*cont.*)
    as oxidizing agent, 13, 17, 18
    study by isotopic carbon, 17
    formic acid formation and, 17
    glutamic acid formation and, 201
    methane formation and, 17
    propionic acid formation and, 18
    succinic acid formation and, 18
    urea formation and, 208
    utilization by bacteria, 17, 157, 158, 560–61, 570–71, 574
      mechanism, 571, 572, 577, 578–79
      photosynthetic, 579
Carbonic anhydrase
  activity of, 13
  constitution of, 13, 58
  distribution of, 13
  estimation of, 58
  inhibition of, 13, 59
  occurrence of, 58, 59
  preparation of, 58, 59
  stability of, 13
  zinc content of, 58
Carboxylase
  activation of, 57
  activity, mechanism of, 56, 58
  diphosphothiamin in, 14
  distribution of, 55
  inhibition of, 56, 57
  instability of, 56
  isolation of, 11
  molecular ratio to diphosphothiamin, 56
  phosphatase hydrolyzing, 57
  Shiff base formation, 15
  of yeast, components of, 54
  zinc and, 56
Carboxypeptidase
  activity
    on acylated amino acids, 37
    dependence on substrate, 36, 37
    determination of, 36
  specificity of, 36
Carcinogenic substances
  cysteine derivatives of, 138
  growth inhibition by, 203, 279
  immunization against, 99
  metabolism of, 279
  spreading of, cholesterol and, 631
Carotene
  determination of, 396
  hibernation and, 397
  iso-, constitution of, 395
  isolation of, 395
  oxidase, 52, 53, 395
  thyroxine inhibition, 287
  utilization of, 192, 397
    disease and, 401
    form of food and, 398
  see also Vitamin A